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Preface

THE second volume of the *World Review of Nutrition and Dietetics* spreads its International net even wider than did Volume I. We have two contributions from the United States, and one each from France, Sweden, South Africa, New Zealand, Switzerland and the United Kingdom, a spread which pretty well encircles the globe.

There has been some telescoping of time as between Volume I and II owing to the various delays which beset the appearance of Volume I. We hope that those who found Volume I of interest and value will likewise find Volume II useful to them.

This preface is being written before we have had time to obtain reactions or receive criticisms of Volume I so that any suggestions made will not have had time to be incorporated in Volume II; however we will be happy to receive suggestions and criticisms and will do our best to incorporate those which we feel will be of advantage to the aims and objects of our publication.

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10th April, 1960

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Proteins and Haematopoiesis

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Contents

I. INTRODUCTION*

The formation of blood cells needs a continuous supply of considerable quantities of nitrogenous material. The latter is indispensable for the synthesis of the nuclear and cytoplasmic proteins and nucleic acids of the erythroblasts and leucocytes, also for the production of haemoglobin, especially for globin, the synthesis of haem needing, in addition to iron, only glycine (Shemin and Rittenberg, 1941) and acetic acid (Bloch and Rittenberg, 1945).

The protein requirements of haematopoiesis should, by themselves, justify a high incidence of anaemia and leucopenia in human malnutrition states. Indeed, such dietary conditions induce regularly a decrease in the total circulating mass of haemoglobin (Walters, Rossiter and Lehman, 1947; Cachera, Lamotte and Dubrisay, 1951) as a consequence of the loss of weight.

However, except in cases with oedema and haemodilution, true anaemia, defined by a decrease in the blood concentration of both erythrocytes and haemoglobin, does not attain a high degree in most of the protein deficiency states (Waitz and Wellers, 1948; Keys *et al.*, 1950). The highest incidence of severe anaemia has been reported during the famine in the ghetto of Warsaw in 1942. This anaemia was microcytic and hypochromic (Szejnman, 1946) and was accompanied by an erythroblastic infiltration of the bone marrow, like the experimental anaemia of protein-depleted rats. Leucopenia was very common, with total disappearance of eosinophils, except in cases of parasitic infestation (Braude-Heller, Rotbalsam and Elbinger, 1946).

The hypochromic type of the anaemia has been reported also by Debray *et al.* (1946) as well as by Mazzoleni and Piemonte (1947), on inmates of German deportation camps. However, according to other investigators (Rossiter, 1946; Lamy, Lamotte and Lamotte-Barillon, 1948) the malnutrition anaemia is ortho- or hyperchromic, whereas Berning (1947) observes both types, hyper- as well as hypochromic ones.

The discrepancy between the conclusions of these different reports seems to be due to the fact that in human beings nutritional anaemia is rarely caused by protein depletion alone, but almost always, although to a various extent, by a concurrent lack of proteins, iron and haematopoietic vitamins. That is why some cases of nutritional anaemia (the majority of the microcytic and hypochromic ones) can be cured by iron alone, whereas other cases such as those of macrocytic and hyperchromic anaemia in sprue, respond very well to folic acid or vitamin B_{12}. In actual fact, both vitamins act, not only by catalyzing certain haematopoietic processes, but also by stimulating the appetite and thereby increasing the protein intake. On the other hand, the hypovitaminosis which accompanies protein-deprivation states, results partly from the inhibition of vitamin synthesis in the digestive tract because of changes in the microflora induced by lack of proteins.

Infectious diseases, malaria and intestinal parasites often play a decisive role in the appearance of anaemia in malnutrition. Thus, in kwashiorkor, a disease induced by protein deficiency, to be found mostly among young children in the tropics, severe anaemia is observed chiefly as a consequence of simultaneous malaria or hook-worm infestation (Lehmann, 1949; Gounelle, 1953).

Deficiency of protein absorption seems also to play a role in hypochromic anaemias observed after gastrectomy (Fauvert, Hartmann and Guenin, 1952) as well as in enterocolitis and dysenteria (Berning, 1947).

In laboratory animals pure protein depletion anaemia is much easier to produce than in humans, by giving synthetic diets exclusively deprived of proteins. Nevertheless, the existence of metabolic interrelations between various amino acids, vitamins and other nutritional factors makes it sometimes impossible to define with precision the degree of responsibility which should be attributed to the absence of one or another of these nutrients in the appearance of blood-cell anomalies.

In this report, we shall first analyse the haematological characteristics of the anaemia and leucopenia induced by experimental protein depletion, then the erythro- and leucopoietic effects of alimentary proteins and the participation in haematopoiesis of tissue reserve proteins and of products of the physiological haemolysis. Then, we shall study the specific action of various amino acids and their interrelations with vitamins, as well as the hormonal control of the haematopoietic utilization of proteins. Finally, some hypotheses concerning the physiological and biochemical mechanisms of the blood anomalies induced by protein depletion will be discussed.

*The survey of literature pertaining to this review was completed in March 1960.

II. CHARACTERISTICS OF ANAEMIA AND LEUCOPENIA INDUCED BY EXPERIMENTAL PROTEIN DEPLETION

Protein depletion induces a rapid drop in total blood volume (Metcoff, Favour and Stare, 1945; Benditt, Straube and Humphreys, 1946), but a very small intake of proteins (about 4 per cent) is sufficient in the rat for the maintenance of a nearly normal concentration of red cells and haemoglobin over a long period of time (Pearson, Elvehjem and Hart, 1937; Orten and Smith, 1937; Orten and Orten, 1943; Aschkenasy and Aschkenasy-Lelu, 1947; Aschkenasy and Benhamou, 1950).

Indeed, Whipple and his collaborators (Whipple, 1942; Whipple and Robscheit-Robbins, 1951) have shown in their experiments on dogs, made anaemic by repeated bleeding and simultaneous protein deprivation, that erythropoiesis belongs to the preferential activities of the organism and that it takes precedence even over production of plasma proteins. Also in the rat, an incomplete deprivation of proteins (for instance, a 4 per cent diet) induces, in the first place, a diminution of the levels of plasma proteins, especially serum albumin, and, only at a later stage, a decrease in the concentration of erythrocytes and haemoglobin (Aschkenasy and Benhamou, 1950).

In fact, diets which are deficient but not entirely lacking in proteins provoke for a long time only a decrease in haemoglobin and haematocrit levels, whereas the erythrocyte counts per cu mm remain unchanged (Orten and Smith, 1937). Consequently, the mean haemoglobin concentration per erythrocyte and the mean corpuscular volume diminish significantly (Aschkenasy and Aschkenasy-Lelu, 1947; Aschkenasy and Benhamou, 1950). On the other hand, an increase in the blood reticulocyte amounts has also been reported in such diets, even in the absence of any change of erythrocyte counts (Orten and Orten, 1943).

The deficiency of the erythropoiesis in diets low in protein is demonstrated particularly by the observation that moderate amounts of different toxic agents are sufficient to produce a significant anaemia if given with a protein poor diet, whereas similar doses are innocuous with a well-balanced diet. Thus, carbon tetrachloride induces anaemia in undernourished rats (Gajdos and Erkeletyan, 1945) but not in rats fed a diet rich in protein (Aschkenasy and Rolland, 1947). The protein intake also controls the haematological effects of sulphonamides (Aschkenasy, Polonovski and Rolland, 1948; Shehata and Johnson, 1948) and of aminoacetonitrile, a compound producing osteolathyrism (Aschkenasy, 1960*a*).

It may be that protein deficiency favours the haemolytic action of the above-mentioned toxic agents either by inducing a structural defect of the erythrocytes or by lowering the levels of some tissular enzymes involved in the detoxifying process.

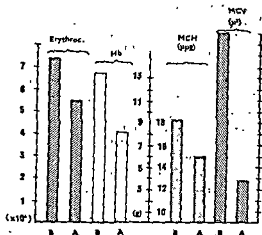


FIG. 1.1. EVOLUTION OF THE BLOOD LEVELS OF ERYTHROCYTES AND HAEMOGLOBIN, OF THE MEAN CORPUSCULAR HAEMOGLOBIN (MCH), AND THE MEAN CORPUSCULAR VOLUME (MCV).
B = before; A = after 30 days of protein starvation.

In the absence of any exogenous intoxication or other noxious influences, it is chiefly with a diet entirely lacking in proteins that a significant anaemia can be easily produced (Aschkenasy, 1944–6). This anaemia is hypochromic and microcytic (Fig. 1.1). During the first days of the protein starvation, before any decrease in haemoglobin concentration, one observes a reduction of the number of blood reticulocytes (less than 1 per cent against 2–5 per cent before the beginning of the diet) but, in proportion, as anaemia progresses the reticulocytosis increases and finally exceeds the normal levels (Aschkenasy, 1944–6). At the same time, growing amounts of erythroblasts appear in the blood. This tendency towards a renewal of the erythrocyte population in the blood at the end stage of protein depletion is somewhat surprising in anaemia due to a lack of haematopoietic materials.

The bone marrow becomes extremely rich in small erythroblasts (normoblasts) (Aschkenasy, 1944–6). As will be seen later, these changes may be attributed either to a maturation block or to an accelerated production of red cells designed to compensate for an excessive haemolysis.

The anaemia is almost always accompanied by leucopenia with a marked decrease in the number of both neutrophils (Aschkenasy, 1944–6; Kornberg, Daft and Sebrell, 1946; Daft, 1947) and lymphocytes

(Aschkenasy, 1944–6) as well as an almost complete disappearance of eosinophils (*ibid.*).

Until a very late stage, the blood disorders induced in the rat by protein depletion can be cured by giving a diet with adequate amounts of a complete protein such as casein. During the recovery, a massive but transitory release of reticulocytes, also of small erythroblasts (normoblasts), into the circulation is observed between the third and the eleventh day. Beyond this 10-day period the levels of all the immature erythrocytes return to their normal proportions (less than 5 per cent), whereas haemoglobin and the number of adult erythrocytes continue to increase (Aschkenasy and Aschkenasy-Lelu, 1947).

The haematocrit and mean corpuscular volume values first show a peak at the moment of the reticulocyte crisis, obviously because of the larger volume of these cells. Later, after a temporary fall, the level of haematocrit increases again following those of haemoglobin and erythrocyte counts. However, if the refection diet does not supply adequate amounts of protein, then the haematocrit remains relatively low, even after normalization of the erythrocyte counts, and thus there is no correction of the microcytosis (Aschkenasy and Pariente, 1951).

The release of reticulocytes regularly coincides with a sharp increase in the number of neutrophils, lymphocytes and eosinophils. This increase is followed by a temporary reduction of the number of these cells and it is only later that the leucocytosis returns progressively to its normal level (Aschkenasy and Aschkenasy-Lelu, 1947; Aschkenasy, 1959*a*).

If the refection diet is deficient in proteins it is unable to restore the animals, which succumb after several days or weeks. During this period the decrease in haemoglobin and erythrocyte counts reaches very low levels, even below those found just before death in rats maintained on a protein-free diet without any attempt at refection. This paraxodical amplification of anaemia, produced by an inadequate refection diet, is accompanied by a marked erythroblastosis and reticulocytosis which increase continuously until the death of the animals, instead of being only transitory as at the beginning of a successful restoration (Fig. 1.2). In the last chapter of this report we shall try to determine to what extent the above-mentioned peculiarities of the protein depletion anaemia should be attributed to a maturation block, and to what extent to haemolysis.

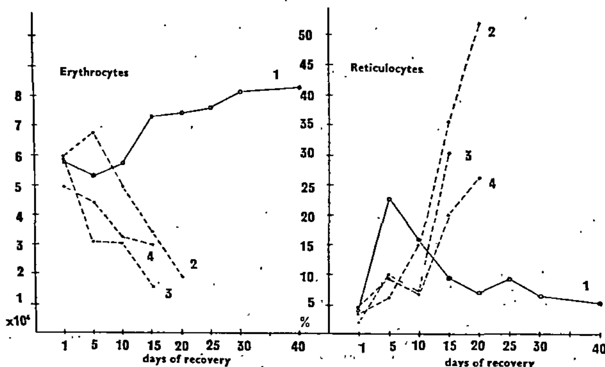


FIG. 1.2. EVOLUTION OF THE BLOOD LEVELS OF ERYTHROCYTES AND RETICULOCYTES IN THE COURSE OF ATTEMPTED CORRECTION OF PROTEIN-DEFICIENCY ANAEMIAS

1 = Successful recovery: the erythrocyte level increases progressively with a reticulocyte crisis on the sixth day.
2, 3, 4 = Unsuccessful recoveries: the anaemia becomes extremely severe while the reticulocytosis increases progressively

III. HAEMATOPOIETIC EFFECTS OF PROTEINS

A. Alimentary Proteins

In dogs made anaemic both by repeated withdrawal of blood and by a diet poor in proteins, not only haemoglobin and globin itself (Robscheit-Robbins and Whipple, 1937), but also casein, liver and muscle proteins are highly haematopoietic (Robscheit-Robbins, Miller and Whipple, 1943). All these proteins regenerate haemoglobin preferentially before plasma proteins: in general the production ratio is of about 2 g haemoglobin to 1 g of plasma proteins for casein and of 3 to 4 g of haemoglobin to 1 g of plasma proteins for striated muscle (meat) (Whipple and Robscheit-Robbins, 1951).

The regeneration of haemoglobin takes precedence over that of plasma proteins even after administration of plasma proteins themselves (Robscheit-Robbins *et al.*, 1943; Whipple and Robscheit-Robbins, 1951). In contrast, plasmopoiesis is favoured by egg-protein and, but less strongly, by lactalbumin (*ibid.*). Supplementing both proteins by digests of other proteins causes a fall in the plasma protein–haemoglobin ratio.

In rats, the haematopoietic potency of certain proteins seems to be somewhat different from that found in dogs. Thus, for Orten and Orten (1946) casein and lactalbumin are at an 18 per cent protein level, of about the same value for haemoglobin regeneration in rats made anaemic by bleeding. On the other hand, according to Buechler and Guggenheim (1949), proteins of eggs and meat, and even those of soya, surpass casein with respect to haemoglobin regeneration in protein-depleted rats.

Human and beef globin are not very efficacious in rats, probably because this protein is poor in isoleucine, which appears to be indispensable for the haemoglobin synthesis in these animals (Orten, Bourque and Orten, 1945).

Among the vegetable proteins, all those which are deficient with respect to one or more of the essential amino acids such as lysine (zein and gliadin) or tryptophan (zein) are, of course, inadequate for haematopoiesis (Gillespie, Neuberger and Webster, 1945). On the other hand, as has been already remarked, Buechler and Guggenheim (1949) found that processed soya had, in the rat, the same anti-anaemic potency as casein.

Systematic comparison between various cereals and pulses has been made recently on young rats from the point of view of growth promotion and haemoglobin and plasma protein production (Chitre and Vallury, 1956). Of the cereals, only wheat approached the value of casein, whereas Bengal gram (the chick pea) was the most effective of the pulses on haemoglobin synthesis although rather poor in promoting plasma protein production. According to these authors the differences of the nutritional values of the vegetables are due chiefly to their content of methionine, lysine and tryptophan.

It goes without saying that the effects of the different animal, as well as plant proteins, depend not only on their amino acid content but also on their amount in the diet. Even a protein low in certain amino acids may be satisfactory when given in quantities sufficient to supply the required amount of the limiting amino acids. Conversely, even proteins of a high haematopoietic value are inadequate if they are given at a level of less than 10 per cent, such a level being too low to meet the requirements for the sulphur amino acids methionine and cystine.

Thus, at a 7 per cent level, casein permits only a very incomplete and slow reparation of the protein-depletion anaemia in the rat, and the reticulocyte crisis is not very marked, whereas the recovery proceeds at a rapid rate with 15 and 30 per cent levels (Aschkenasy and Aschkenasy-Lelu, 1947).

On the whole, it seems that whenever a protein is adequate for growth and weight maintenance, either because of its balanced amino acid composition or of its high levels in the diet, it is also effective on haematopoiesis.

Therefore, it can be presumed that the different factors which are able to change the biological value of a protein, such as cooking and other methods of processing, as well as digestibility and the time of ingestion (i.e. simultaneously or alternately with other nutrients), may also modify the haematopoietic effects of the latter.

On the other hand, it is obvious that simultaneous feeding with different, even inadequate, proteins is able to bring about a satisfactory rate of haematopoiesis by reciprocal compensation of their individual deficiencies conditioned by low amounts of certain essential amino acids.

B. Tissular Proteins and Catabolic Products of Haemolysis

Even after the complete removal of alimentary proteins, the synthesis of haemoglobin continues (though at a slow rate) for a certain time, on account of the transfer of nitrogenous materials from protein reserve stores. Robscheit-Robbins and Whipple (1935) have shown that whenever a dog receives, for a limited period, a diet rich in proteins, it utilizes the latter, not only for new haemoglobin production, but also for accumulation of protein reserve stores, which permit the protracted maintenance of a normal

haematopoiesis during a subsequent protein deficient diet. These reserve stores are even sufficient to permit an increase in the blood (red) cell production in protein-starved animals in response to certain stimuli such as cobalt (Orten and Orten, 1945) or dinitrophenol (Aschkenasy, 1959*b*).

The protein reserve available for haematopoiesis consists of non-specific materials which, although easy to mobilize, adopt the histological structure of the tissues in which they are located: in the first place, the liver (Robscheit-Robbins and Whipple, 1935; Addis, Poo and Lew, 1936) but also the striated muscles, and the spleen which undergoes an early atrophy in protein starvation (Aschkenasy, 1944–46). In addition, it is noteworthy that the intestinal mucosa secretion proteins, which are partly reabsorbed by the intestine and re-utilized for metabolic purposes, should also be considered as reserve proteins (Nasset, 1957).

It is the endocrine glands which seem to play the essential role in the distribution of alimentary, as well as tissue reserve proteins among the various requirements of the animal organism, including the haematopoietic function.

The rate of destruction of blood cells being equal to the rate of their production in a physiological equilibrium state, the products of disintegration of these cells should be sufficient by themselves to satisfy the protein needs for haematopoiesis.

In actual fact, all these products return to the common pool of labile tissue proteins and are then utilized, concurrently with other nitrogenous metabolites of tissular or exogenous origin, for various requirements of the body. Apparently, there is no preferential utilization of the products of haemolysis for haematopoiesis (Madden *et al.*, 1945; Yuile, 1949). It has also been suggested that these catabolites might enhance the production of some plasmatic haematopoietin (Nizet, 1955). However, only an inconstant increase in such a factor has been found in human haemolytic anaemias (Gurney, Jacobson and Goldwasser, 1958; Gordon, 1959).

IV. HAEMATOPOIETIC EFFECTS OF VARIOUS AMINO ACIDS

A. Erythropoietic Effects of Amino Acids

Mixtures containing the 10 amino acids indispensable for the growth of rats (Rose, 1938), in proportions existing in casein, restore the serum proteins as well as the erythrocyte levels to the same extent as casein does, if they are given for 10 days to protein-depleted rats (Benditt *et al.*, 1947). On the other hand, however, if the experimental period is more protracted (27 days), casein appears to be more effective in respect to erythropoiesis (as well as to the weight recovery) than an acid hydrolysate of the latter supplemented with both cystine and tryptophan (Bourgeat and Aschkenasy, 1948). It may be that this result is to be attributed rather to a toxic effect of this preparation than to its deficiency in some anabolic (and haematopoietic) factor such as the hypothetical "strepogenin" (Woolley, 1946).

According to Benditt *et al.* (1947), even mixtures containing only 9 amino acids, without arginine (tryptophan, lysine, methionine, histidine, threonine, valine, leucine, isoleucine and phenylalanine) are sufficient for plasma and erythropoiesis in short-time recovery experiments. On the other hand, mixtures lacking only one of these 9 amino acids are inadequate.

This latter result may be due, not only to a specific requirement for the absent amino acid, but also to a decreased intake of the remaining amino acids and other nutrients, on account of the loss of appetite induced by the nutritional imbalance (Rose, 1938; Frazier *et al.*, 1947).

It is, therefore, obvious that a single essential amino acid given alone is unable to counteract protein depletion anaemia (Orten and Orten, 1945). Consequently, the haematopoietic potency of any one of the amino acids cannot be demonstrated without the supplying of other amino acids, or of proteins, as will also be seen from certain experiments which we shall now consider.

1. Methionine

(a) *Specific Deprivation of Methionine*

Young rats fed with all essential amino acids, except for methionine, exhibit anaemia which can be prevented by very small amounts of this amino acid (8 mg/rat/day) whereas liver necrosis, induced by the same diet, is counteracted only by tenfold higher doses of methionine (Glynn, Himsworth and Neuberger, 1945).

Hypochromic anaemia is also observed by Albanese, Holt Jr., Irby and Brumbach Jr. (1946) in rats exclusively deprived of methionine. On the other hand, protein starvation anaemia cannot be cured in rats by an amino acid mixture lacking methionine (Benditt *et al.*, 1947).

Finally, according to Spisni (1950), the hypochromic anaemia produced in rats by a goat milk diet is due to the low amount of sulphur amino acids in this diet.

(b) *Effects of Methionine in General Protein Deficiency*

In rats on a protein-free diet, methionine alone does not prevent the appearance of anaemia, whereas it

partly counteracts the decrease in plasma proteins, particularly in serum-albumin levels (Aschkenasy, Boissier and Rolland, 1949). The same contrast between the low erythropoietic efficiency of methionine and its striking action on plasma proteins (with specific effect on albumin as well as on α- and β-globulins) has also been found in a diet including 4 per cent casein (Aschkenasy and Benhamou, 1950).

On the other hand, methionine significantly enhances erythropoiesis if the diet contains 7 per cent protein (casein). Such a beneficial effect has been observed in normal growing rats (Aschkenasy and Benhamou, 1952) as well as in adult rats made previously anaemic by prolonged protein starvation (Aschkenasy and Pariente, 1950–1). In both experimental conditions the formation of haemoglobin takes precedence over the increase in the number of erythrocytes. Methionine also enlarges simultaneously the mean corpuscular volume, which remains subnormal if the 7 per cent refection diet is not supplemented with this amino acid.

(c) *Effects of Methionine in Some Toxic Anaemias*

A protective effect of methionine has been reported in rats made anaemic by carbon tetrachloride (Gajdos and Erkeletyan, 1945), sulphadiazine (Aschkenasy, Polonovski and Rolland, 1948) or phenylhydrazine (Bénard *et al.*, 1948). In sulphonamide intoxication

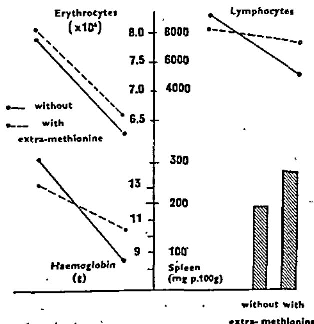


Fig. 1.3. Action of Methionine (0·3g/100 g of dry ration) on Erythrocytes, Haemoglobin and Lymphocytes and on Spleen Weight in Rats given a Protein-poor Diet (4 per cent) with added Sulphadiazine (0·4 per cent) for 100 Days

methionine prevents the decrease in haemoglobin much more than the decrease in the erythrocyte count (Fig. 1.3). However, such an effect appears only if the basal diet is deficient in proteins and, therefore, lacking in methionine. It is probably the methionine content of casein which explains the protective potency of this protein against the toxic effects of sulphonamides (Wright, Skeggs and Sprague, 1945) as well as of carbon tetrachloride (Aschkenasy and Rolland, 1947).

It is possible that methionine counteracts the anaemia induced by various toxic agents by increasing the cellular content of sulphur-containing enzymes and peptides such as glutathione. The extreme susceptibility of protein-depleted animals to different toxins might perhaps be explained by the well-known diminution of these activators in the blood and tissues following protein deprivation (Miller, 1944; Leaf and Neuberger, 1947). However, there is also the possibility that the beneficial effect of methionine might be attributed to its anabolic action on the liver and on the plasma proteins, an action capable of enhancing the detoxifying mechanisms.

On the other hand, methionine has been found capable of neutralizing even *in vitro* the bacteriostatic activity of these drugs (Kohn and Harris, 1941).

(d) *Conclusion*

To sum up: (1) Methionine displays a significant erythropoietic potency, only if its intake in the basal diet is inadequate. (2) It increases the haemoglobin level much more than the number of erythrocytes, thereby causing an augmentation of the mean corpuscular volume and of the mean corpuscular concentration of haemoglobin. (3) In contrast to its erythropoietic activity, the beneficial effect of methionine on plasma proteins does not need concurrent administration of proteins or other amino acids, as it is observed even on a protein-free diet.

2. Cystine

Deprivation of cystine alone does not induce anaemia in rats and this amino acid cannot prevent anaemia due to an exclusive lack of methionine (Glynn *et al.*, 1945).

Like methionine, cystine is much more effective on plasma proteins than on blood-cell production in protein-deficient rats (Aschkenasy and Benhamou, 1950). This effect is certainly related to the high amount of cystine in serum-albumin (Block and Bolling, 1947).

On the other hand, cysteine and cystine appear to protect against anaemia and leucopenia induced in rats by X-radiations (Patt, Smith and Jackson, 1950; Rosenthal, Goldschmidt and Pickering, 1951) or by

nitrogen mustard (Weisberger and Heinle, 1950; Weisberger, Heinle and Levine, 1952). No investigations have yet been made concerning protection by cystine against other blood-destroying agents.

3. Tryptophan

Diets devoid of tryptophan induce in rats a hypochromic anaemia as well as a diminution of plasma proteins (Albanese *et al.*, 1943).

Likewise, amino acid mixtures lacking tryptophan are not adequate for the cure of experimental anaemia of rats due to protein depletion (Benditt *et al.*, 1947) or bleeding (Sebrell, 1949).

Conversely, a beneficial effect of this amino acid has, for a long time, been reported in rats made anaemic by bleeding or by phenylhydrazin (Okagawa and Tatsui, 1931; Yeshoda and Damodaran, 1947) and even in non-anaemic animals (rabbits and dogs) (Fontès and Thivolle, 1930).

An inborn error in tryptophan metabolism has been demonstrated in children suffering from congenital hypoplastic anaemia (erythrogenesis imperfecta). However, the urinary excretion of anthranilic acid, which is an abnormal intermediary metabolite of tryptophan, can be reduced in this disease by giving massive doses of riboflavin without any concurrent change in the haematological status (Altman and Miller, 1953).

4. Histidine

Like tryptophan, histidine favours the reparation of haemorrhagic or protein-starvation anaemias of rats (Sebrell, 1949; Benditt *et al.*, 1947). Addition of histidine to egg protein, which is rather poor in this amino acid, causes in doubly-depleted dogs (anaemic plus hypoproteinaemic) a fall in the plasma proteins to haemoglobin ratio from 90 to 100 per cent toward the common protein ratio of 50 per cent (Whipple and Robscheit-Robbins, 1951).

Conversely, a diet devoid of histidine provokes in rats a moderate loss of haemoglobin (Fontès and Thivolle, 1930; Maun, Cahill and Davis, 1946; Fuller, Neuberger and Webster, 1947; Nasset and Gatewood, 1954).

In view of the considerable proportion (8 per cent) of this amino acid in globin (Block and Bolling, 1947) the reported results are not at all surprising, at least in the rat which, in contrast to man (Rose *et al.*, 1951), seems to be incapable of synthetizing the iminazole ring (Moore and Wilson, 1957).

5. Phenylalanine and Tyrosine

Whipple and Robscheit-Robbins (1937) report an increased production of haemoglobin in dogs, rendered anaemic by bleeding, after administration of phenylalanine and tyrosine. Conversely, a diminution of haemoglobin is noted by Maun, Cahill and Davis, (1945*a*) in rats deprived of both amino acids.

However, Winnick, Friedberg and Greenberg (1948) do not discover any radioactivity in erythrocytes of rats injected intravenously with ^{14}C-tyrosine.

It is to be noted that human pernicious anaemia is accompanied by alteration of the catabolism of phenolic amino acids, as shown by the augmentation of phenols in the blood serum and in the urine (Swendseid, Burton and Bethell, 1943), as well as by troubles of pigmentation (vitiligo, canitia), which seem to be related to a defect in the synthesis of melanine from tyrosine. Liver extract cures all these troubles as well as anaemia itself.

6. Leucine

The high amount of this amino acid (16 per cent) in haemaglobin (Block and Bolling, 1947) explains the avidity with which the bone-marrow cells incorporate labelad leucine *in vitro* (Borsook *et al.*, 1950), also the efficiency of this amino acid in protein depletion or bleeding-induced anaemias in the rat (Benditt *et al.*, 1947; Sebrell, 1949). However, neither Maun *et al.* (1945*b*) nor Bowles *et al.* (1949) observe anaemia in rats exclusively deprived of leucine.

7. Isoleucine

Orten, Bourque and Orten (1945) and Orten and Orten (1946) have shown that beef and human globin were inadequate for haemoglobin regeneration in rats, but that their efficiency could be improved by the addition of isoleucine, which, according to these authors, permits the synthesis of a metabolic precursor of rat globin.

Isoleucine has been found to be useful in the treatment of anaemias induced in the rat by protein depletion (Benditt *et al.*, 1947) as well as by bleeding (Sebrell, 1949; Sebrell and McDaniel, 1952) or intoxication (Chandran and Damodaran, 1951).

8. Lysine

Proteins devoid of lysine (gliadin and zein) do not permit the maintenance of a normal erythropoiesis in the rat (Harris, Neuberger and Sanger, 1943; Gillespie, Neuberger and Webster, 1945). Bevan *et al.* (1950) also found that lysine-deprived rats eat less, lose weight, and have lower levels of haemoglobin.

On the other hand, haemorrhagic anaemia of the rat cannot be cured by an amino acid mixture lacking lysine (Sebrell, 1949).

The low content of lysine in most vegetable proteins except yeast (Block and Bolling, 1947) is

probably the major cause of their haematopoietic inadequacy.

9. Threonine and Valine

The presence of both amino acids in the diet is also a necessary condition for the refection of nutritional (Benditt *et al.*, 1947) or haemorrhagic anaemias (Sebrell, 1949) in the rat.

10. Conclusion Concerning the Erythropoietic Action of Amino Acids

In fact, all amino acids which are essential for growth and weight maintenance, are also indispensable for erythropoiesis. Among the semi-essential amino acids (histidine, tyrosine and arginine) only the first two seem to be necessary in the dog and the rat, whereas arginine is superfluous, perhaps because of the very small amount of this amino acid in haemoglobin (Block and Bolling, 1947). According to Bowles *et al.* (1949) it is even noxious for erythropoiesis in the rat.

It is noteworthy, however, that reticulocytes of blood taken from protein depleted rats are unable *in vitro* to transform themselves into adult erythrocytes unless the medium is supplemented with 11 amino acids, including, not only all the 9 essential ones and arginine, but also glycine (Nirzet, 1951). Apparently, the need for glycine is justified by its participation in the synthesis of haem, which can be carried out by reticulocytes, even *in vitro* (Shemin and Rittenberg, 1945), whereas glycine itself can be synthetized only *in vivo* but not *in vitro*.

On the other hand, Nizet and Lambert (1954) have shown that reticulocytes can synthetize haem and globin, not only from free amino acids (in their isomeric form L), but also from di- and tripeptides. However, the incorporation of the latter is not as rapid as that of amino acids.

In vivo synthesis of haemoglobin seems to utilize free amino acids, which are carried by the immature red cells themselves. Indeed, Piha (1954) has demonstrated that reticulocytes contain more free amino acids than mature erythrocytes.

In so far as we can say at the present time, each anaemia induced by selective deprivation of any amino acid seems to be of the same hypochromic type as anaemia following a complete protein depletion. Therefore, a lack of amino acid material appears to be more detrimental for the synthesis of haemoglobin than for the multiplication of erythrocytes themselves and the formation of their stroma.

Conversely, whenever an amino acid such as methionine is effective in the treatment of any anaemia, in human beings (Layani *et al.*, 1949) as well as in animals—for instance in rats intoxicated with sulphadiazine (Aschkenasy *et al.*, 1948) or deprived of proteins—it is always much more the haemoglobin than the erythrocyte count which benefits by this action.

B. Leucopoietic Effects of Amino Acids

Kornberg (1946) has shown that protein depletion induced agranulocytosis could be counteracted in rats by feeding, either an amino acid mixture, or casein, provided the animals simultaneously received folic acid.

The specific action of individual amino acids on leucopoiesis has not been the subject of much experimental work up until now.

According to Bowles *et al.* (1949), there is a moderate decrease in the leucocyte count in young rats selectively deprived of histodine, leucine, lysine, methionine or tryptophan. On the contrary, leucocytosis increases after deprivation or arginine.

On the other hand, specific deprivation of various amino acids induces atrophy of thymus, as does a protein-deficient diet (Aschkenasy, *1944–6*); phenylalanine (Maun *et al.*, *1945a*; Scott, Schwartz and Ferguson, *1950*); threonine (Scott and Schwartz, 1953); histidine (Maun, Cahill and Davis, 1946; Scott, 1954); tryptophan (Scott, 1955); isoleucine (Scott, 1956).

However, contrary to what occurs in protein depletion, involution of spleen or lymph nodes has been reported up until now in only a few cases of specific amino-acid deficiencies, perhaps because the experiments have not yet been carried far enough.

Methionine seems to be particularly useful for leucopoiesis. Deprivation of this amino acid induces in the rat, not only anaemia but also lymphocytopenia (Dinning, Payne and Day, 1951). On the other hand, methionine reduces the frequency of leucopenias in protein-deficient rats exposed to benzene (Li and Freeman, 1947). In rats made leucopenic by prolonged protein starvation, addition of methionine to a refection diet accelerates the reconstitution of the lymphoid organs and of the blood levels of lymphocytes (Aschkenasy and Pariente, 1951). Finally, in rats intoxicated by sulphadiazine, and receiving a diet deficient in proteins, supplementation with methionine counteracts the lymphocytopenic effect of the toxic factor as well as the involution of the spleen (Aschkenasy *et al.*, 1948 (*see* Fig. 1.3).

In the two preceding experiments (1948, 1951) the methionine-treated as well as the control rats received adequate amounts of choline in their basal diet. Nevertheless, the beneficial action of methionine was possibly due, not only to its sulphur component but

also to its methyl group, the metabolic pathways of which are not entirely similar to those of the methyl groups of choline. However, an intervention of the sulphur group would agree with the findings that X-radiation and nitrogen-mustard-induced leucopenias can be prevented by cystine and cysteine (*c.f.* above).

V. INTERRELATIONS BETWEEN AMINO ACIDS AND VITAMINS

A. Tryptophan and Niacin

Anaemias normally attributed to a deprivation of amino acids may perhaps be due to the lack of vitamins derived from the former.

Thus, deprivation of tryptophan induces a lack of niacin which may itself intervene in the maturation process of erythroblasts through the pyridine co-enzymes of these cells. Indeed, protein-depletion anaemia and leucopenia are both considerably reduced in the rat by an alimentary excess of niacin (Aschkenasy, 1947). In rats receiving a protein-rich diet, nicotinic avitaminosis cannot be realized because of the synthesis of niacin by the intestinal microflora. On the contrary, such a synthesis seems to be lacking in protein-deficiency states. That is probably why exogenous niacin alone, given at high doses, is able to compensate for the deficiency of endogenous vitamin as well as of tryptophan.

Conversely, anaemia induced in the pig by niacin deficiency can be prevented by an increase in the intake of proteins, which act, probably, through their tryptophan content (Wintrobe *et al.*, 1945).

B. Tryptophan and Pyridoxine

The metabolic conversion of tryptophan to nicotinic acid is controlled by pyridoxine (Snell, 1953). Nevertheless the mechanism of anaemia due to a deficiency of tryptophan is apparently not the same as that of anaemia due to avitaminosis B_6: according to Cartwright *et al.* (1945) only the latter is accompanied by hypersideraemia and haemosiderosis; on the other hand, pyridoxine-deficiency anaemia does not respond to tryptophan (Cartwright, Wintrobe and Humphreys, 1944).

C. Methionine and Thiamine

Another example of haematopoietic interactions between vitamins and amino acids is the antagonism thiamine-methionine observed in protein-depleted rats.

In such animals, administration of considerable doses of thiamine increases the fall of the erythrocyte levels (Aschkenasy, 1947) as well as that of plasma proteins (Aschkenasy, Boissier and Rolland, 1949) and of tissular proteins (Boissier and Aschkenasy, 1950). Now, all these noxious effects are counteracted by methionine (Aschkenasy, 1947; Aschkenasy *et al.*, 1949; Boissier and Aschkenasy, 1950).

D. Methionine and Pantothenic Acid

Dinning, Neatrour and Day (1954) protect rats by methionine against lymphocytopenia induced by deprivation of pantothenic acid. According to these authors (1955) the amino acid contributes to the synthesis of co-enzyme A, as the precursor of its thioethylamine fraction.

E. Amino Acids and Folic Acid

As has been already pointed out, removal of proteins from diet causes radical quantitative and qualitative changes in the synthesis of vitamins by the intestinal bacterial flora. Folic acid seems to be one of the vitamins which are not produced in adequate amounts in such deficiency conditions. That would explain the observation of Daft (1947), that rats rendered agranulocytic by a protein-free diet give a prompt granulocytic response when they receive folic acid, whereas the response is rather slow to a mixture of amino acids without this vitamin. Nevertheless, according to Kornberg (1946) neither amino acids nor folic acid alone are able to cure protein-depletion granulocytopenia. The latter can be counteracted only by a simultaneous administration of both nutrients.

On the other hand, folic acid increases notably the anti-anaemic as well as the anti-leucopenic effects of casein in rats fed a refection diet after a protracted protein starvation (Aschkenasy and Aschkenasy-Lelu, 1947; Aschkenasy, Aschkenasy-Lelu and Rolland, 1949*a*).

F. Methionine and Vitamin B_{12}

Like folic acid, vitamin B_{12} intervenes in certain stages of the protein synthesis in the haematopoietic tissues. One of its actions is related to the synthesis and metabolism of methyl groups, which explains the possibility of a partial replacement of methionine by vitamin B_{12} in some physiological processes. Thus, in experimental leucopenia, produced by a lack of labile methyl groups, vitamin B_{12} and betaine, given concurrently, can replace methionine in respect of its methylating action, and restore the leucopoiesis, whereas betaine alone is not efficacious (Dinning *et al.*, 1950). On the other hand, it is noteworthy that both methionine and vitamin B_{12} enhance the restoration of liver and lymphoid organs (chiefly thymus) as well as that of the blood lymphocytosis in protein-

depleted rats receiving a 7 per cent protein refection diet (Aschkenasy and Pariente, 1950–1).

However, in the same rats, the addition of methionine to the diet is insufficient to replace vitamin B_{12} in regard to erythropoiesis, and the opposite is also true. Indeed, the reticulocyte release is higher with the vitamin than with the amino acid; on the other hand, only the latter increases the mean corpuscular volume and thereby suppresses the microcytosis induced by the protein depletion. Finally, the haemoglobin levels increase much more when both vitamin B_{12} and methionine are given concurrently than if only one of these nutrients is administered (Aschkenasy and Pariente, 1950–1).

Indeed, the haematopoietic potency of vitamin B_{12} involves several other mechanisms in addition to transmethylation. Thus, the vitamin seems to act as a co-factor in the incorporation of amino acids into proteins (Wagle, Mehta and Johnson, 1957), also in the synthesis of nucleic acids (Rose and Schweigert, 1952; O'Dell and Bruemmer, 1957).

VI. INFLUENCE OF CARBOHYDRATES ON THE HAEMATOPOIETIC VALUE OF ALIMENTARY PROTEINS

With a refection diet including no more than 7 per cent casein, the reparation of protein-depletion anaemia is significantly accelerated in the rat by giving starch in place of sucrose in the diet. On the other hand, if the intake level of casein is 15 per cent, then the refection rate of the anaemia is similar with both carbohydrates (Aschkenasy, Aschkenasy-Lelu and Rolland, 1949*b*). Apparently, the increase of protein intake renders superfluous the protein-sparing action exerted by the dietary starch. The latter acts, probably, by stimulating the intestinal microflora capable of synthetizing some anabolizing factor, vitamin or amino acid.

VII. INTERRELATIONS BETWEEN PROTEINS AND HORMONES IN HAEMATOPOIESIS

Proteins and their component amino acids contribute to the maintenance of the blood-cell equilibrium, not only because they belong to the building materials of these cells, but also because they influence certain endocrine secretions controlling the blood-cell pattern.

On the other hand, the endocrine glands seem to be *the principal regulating factors in the haematopoietic* utilization of alimentary and endogenous proteins.

A. Role of Endocrine Imbalance in the Development of Anaemia and Leucopenia Induced by Protein Depletion

These blood disorders are partly conditioned in the male rat by a hypoactivity of the thyroid and testicles, as well as by a relative hyperactivity of the adrenal cortex.

1. Hypoactivity of Thyroid and Testicles

Protracted protein starvation induces a decrease in the secretion of the thyrotropic (Aschkenasy, Benhamou and Rolland, 1952) and the gonadotropic hormones (Aschkenasy, 1953) as thereby an involution of the thyroid and gonads.

Thyroidectomy accelerates the appearance of the anaemia (Aschkenasy and Aschkenasy-Lelu, 1949; Aschkenasy and Puyo, 1952; Aschkenasy and Pariente, 1953), whereas injections of thyroxine, on the contrary, prevent or, at least, delay markedly the anaemia (Aschkenasy, 1954) if they are performed since the beginning of the protein deprivation.

On the other hand, some haematological peculiarities of the anaemia are amplified by thyroidectomy (for instance the medullary erythroblastosis) or orchidectomy (the microcytosis) and, conversely, reduced *by administration of corresponding hormones (chiefly* the erythroblastosis by thyroxine). Thyroxine also prevents the temporary fall of the reticulocyte level at the first stage of protein starvation (Aschkenasy, 1960*b*).

Protein-depletion leucopenia is also generally more pronounced in thyroidectomized rats (Aschkenasy and Aschkenasy-Lelu, 1949), and the involution of lymphoid organs is more advanced in such animals than in controls (Aschkenasy and Pariente, 1953).

On the contrary, thyroxin injections tend to prevent neutro- and lymphocytopenia as well as the involution of lymphoid organs (Aschkenasy, 1954).

2. Hyperactivity of Adrenal Cortex

A hyperactivity of the adrenal cortex is suggested by the following findings: (*a*) Adrenals of protein-depleted rats are relatively larger than those of normal rats and show an increase of the cortex (Aschkenasy, 1953). (*b*) It is almost impossible to keep adrenalectomized rats alive, on a diet very poor in proteins, for more than a few days even if they are given saline

drink; but if such rats receive cortisone, they resist protein starvation for a long time, just as intact rats do (Aschkenasy, 1955).

On the contrary, on an 18 per cent protein diet with saline water, adrenalectomized rats remain alive without cortisone.

These results suggest that glucocorticoid requirements are relatively higher in protein-deprived than in well-nourished rats.

The adrenocortical activity which tends to satisfy the increased need for these hormones probably plays a decisive role in the appearance of the dietary lymphocytopenia. Indeed, not only is the latter prevented by adrenalectomy but, in addition to that, the few adrenal deprived rats remaining alive after some weeks of protein deprivation, exhibit an increase in the lymphocyte levels, which is much stronger than the increase observed after the same intervals in adrenalectomized rats submitted to a balanced diet (Aschkenasy, 1955). This kind of reversed-alarm reaction has been noticed much more in females than in males.

Much more doubtful is the role played by a relative hypercorticism in the appearance of neutro- and eosinopenia. Indeed, although administration of cortisone to protein-depleted rats increases the loss of eosinophils, as well as that of neutrophils (Aschkenasy, 1953), nevertheless, adrenalectomy performed before beginning the diet does not influence markedly the development of either the hypogranulocytosis (Aschkenasy, 1956) or the eosinopenia (Aschkenasy, 1955).

B. Effect of Hormones on the Recovery from Protein Depletion-induced Blood Disorders

The rate of the refection of anaemia and leucopenia can also be influenced, to a large extent, by altering the endocrine status of the animals.

Thus, thyroidectomy generally inhibits any reparation of anaemia if the refection diet is relatively poor in protein (7 per cent) (Aschkenasy and Aschkenasy-Lelu, 1949). Conversely, with the same diet, injections of thyroxine accelerate markedly the regeneration of erythrocytes and eosinophils (Aschkenasy and Dray, 1953–4).

On the other hand, injections of testosterone propionate activate erythropoiesis (Fig. 1.4) and the regeneration of neutrophils, but inhibit that of eosinophils. Recovery of erythropoiesis is also stimulated by ACTH. Oestrogens are devoid of any noticeable effect.

The somatotropic hormone (STH) is noxious for the weight and blood refection if given in large doses (10 rat units per day) with a protein poor diet (7 per cent casein), but, on the contrary, the hormone is moderately beneficial to the red cells if the doses are

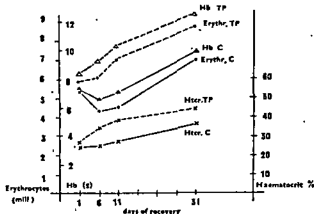


FIG. 1.4. REPARATION OF ANAEMIA (ERYTHROCYTES, HAEMOGLOBIN AND HAEMOTOCRIT) IN RATS RECEIVING A REFECTION DIET POOR IN PROTEIN (7 PER CENT) AFTER 80 DAYS OF PROTEIN STARVATION

Comparison between rats injected with testosterone propionate (TP) and controls (C) injected with olive oil.

very small (1 and 2 rat units per day), whether the diet is rich or poor in protein (18 or 7 per cent) (Aschkenasy and Dray, 1953–4; Aschkenasy, 1959*a*). The favourable action of these doses of STH is not at all amplified by simultaneous injections of insulin, the effects of which have been investigated by us (Aschkenasy, 1959*a*) in order to verify the hypothesis of a synergic action of both hormones.

In summary, all the above-mentioned experiments show the close interdependency between the haematological and the hormonal status of protein-deprived rats. However, they are not by themselves sufficient to warrant the conclusion that either the one or the other of the investigated endocrine glands plays a determining role in the production of anaemia and leucopenia.

Indeed, the effects of each hormone appear to be only fragmentary, and sometimes they are even antagonistic with respect to the different kinds of blood cells. Thus, injections of testosterone propionate protect against anaemia but aggravate the involution of the lymphoid organs and the lymphocytopenia of protein-depleted rats.

On the other hand, neither is the enhancing effect of certain hormones on the refection of the protein depletion-anaemia and leucopenia sufficient to prove that these disorders are caused by a hormonal imbalance.

C. Role Played by Endocrine Glands in the Action of Proteins and Amino Acids on Leucocytes in Normal Dietary Conditions

The intervention of the adrenal cortex in the eosinopenic effect of certain proteins and amino acids

is virtually the only subject which has been investigated in this field up until now.

1. Oral administration of casein brings about, within 4 hours, a fall in the blood eosinophil level, whereas gelatin is without any effect (Vartiainen and Apajalahti, 1953). The action of casein has been attributed to the presence of tyrosine, the latter being also eosinopenic by itself.

Góth *et al.* (1955) report that ingestion of egg white also induces a moderate eosinopenia which, however, does not appear in patients with Addison's disease. The blood serum of persons having consumed ovalbumin, exhibits itself an eosinopenic potency when it is injected into rats, even if the latter are adrenalectomized. Serum taken from control subjects is ineffective. According to Góth *et al.*, ingestion of ovalbumin acts as an alarm stimulus and increases the blood level of glucocorticoids.

The same workers observe also a significant eosinopenia in rats after administration by stomach tube of certain amino acids, especially leucine and methionine. Once more, the results are negative in adrenalectomized rats. Thus, a single feeding with proteins or amino acids seems to be sufficient to stimulate the hypophyso-adrenal system.

2. By intraperitoneal route, serum albumin, γ-globulin and several amino acids also provoke an eosinopenic reaction. A reduced response is observed even in adrenalectomized rats and, therefore, this response appears to be partly independent of a discharge of ACTH (Aschkenasy, 1957, 1958).

In contrast to a single administration, repeated injections of foreign proteins induce an increase in blood eosinophils, probably through an allergic reaction. On the other hand, in protein-depleted rats receiving a refection diet, eosinopenia disappears earlier if this diet is rich than if it is poor in casein (Aschkenasy*).

In conclusion, the above experiments show that protein and amino acid ingestion can notably influence the blood eosinophil levels, and that proteins act in an opposite way, according to the duration of their administration. Thus, the well-known instability of blood eosinophilia seems to be partly conditioned by the protein composition of the diet, chiefly through the mediation of the hypophyso-adrenocortical system.

It appears highly probable that a hormonal imbalance also plays a role in the changes of blood levels of neutrophils and lymphocytes induced by variations in the protein intake even in the conditions of a normal alimentation. However, conclusive evidence for this hypothesis is still lacking up to the present time.

VIII. PATHOGENESIS OF THE BLOOD DISORDERS INDUCED BY PROTEIN DEPLETION

A. Pathogenesis of the Protein-depletion Anaemia

Two anomalies of the turnover of erythrocytes seem to be responsible for this anaemia: (1) a maturation block of the red cells in the bone marrow; (2) an excessive haemolysis.

1. The Role of the Maturation Block

A maturation blockage has already been suggested by Miller *et al.* (1949), who have shown that after administration of ^{14}C-labelled lysine, the isotopic carbon appears, in blood erythrocytes, at a later stage in protein-deficient dogs than in normal dogs.

On the other hand, Bethard *et al.* (1958) have recently reported that protein-deprived rats utilize for erythropoiesis only a very small part of ^{59}Fe injected 5 days before. Thus, 10 days after the removal of proteins from the diet, rats show, in their circulating erythrocytes, only 5 per cent of the injected radioiron dose, whereas the average amount attains 71 per cent in non-deficient rats.

Thus, the maturation block of erythroblasts appears at a very early stage of protein deprivation, obviously a long time before the depletion of the tissue protein stores. It is in the same period that one usually observes a decrease in the blood reticulocyte counts which drop in adult rats from 2 to 4 per cent before the diet to less than 1 per cent (Aschkenasy, 1944–6).

It is worthy of note that a certain parallelism between the erythrocyte radioiron uptake and the level of blood reticulocytes has been found also by Jacobson *et al.* (1957) in rats and mice injected with erythropoietic preparations.

The fact that the peripheral haemoglobin and the erythrocyte counts still remain normal for a long time after the appearance of the maturation block may probably be explained by the decrease in the total erythrocyte mass related to the loss of body weight. Indeed, the diminution of the need for haemoglobin permits the maintenance of a normal concentration of the latter within the circulation in spite of its reduced production.

Some considerations suggest that there is not only one but at least two blocks, one of which interferes with the maturation of erythroblasts and the other with that of reticulocytes.

* Unpublished.

(a) *The Erythroblast Maturation Block*

This block is responsible for the accumulation of small normoblasts in the bone marrow. Its removal is apparently at the origin of the mass release of reticulocytes into the blood, observed in the first days of a refection diet. Besides, the resumption of protein feeding not only elicits a maturation of erythroblasts in the bone marrow but causes also a temporary passage of these immature cells into the peripheral blood, this erythroblastic discharge being observed concurrently with that of reticulocytes (Aschkenasy and Aschkenasy-Lelu, 1947).

Given that the erythroblastic block occurs at the moment of haemoglobin synthesis, it is not surprising that some of its effects such as microcytosis and hypochromia are similar to those induced by iron deficiency. Moreover, the inhibition of haemoglobin synthesis may be reinforced by some disturbances of the iron metabolism such as a lack in siderophilin (Ventura, 1959). It is noteworthy that in contrast to the macrocytosis of vitamin-B_{12} deficiency in pernicious anaemia, the microcytosis of protein deficiency concerns only advanced erythroblasts (normoblasts) and mature erythrocytes and not at all the granulocytes (unpublished).

Although the maturation blockage due to protein deprivation apparently takes place at a later stage than the blockage induced by a lack of vitamin B_{12}, nevertheless the latter facilitates the maturation of normoblasts in protein-depleted animals on condition that they receive concurrently a subnormal protein intake in their refection diet. Indeed, as stated previously, a refection diet containing only 7 per cent casein, provokes a more pronounced reticulocyte crisis if it is supplemented with vitamin B_{12} than without the latter. On the other hand, antipernicious liver extracts are completely ineffective in rats against protein-depletion anaemia in the absence of any protein intake (Aschkenasy and Aschkenasy-Lelu, 1947).

(b) *The Reticulocyte Maturation Block*

This second block concerns the passage of erythrocytes from the reticulocyte stage to the mature end stage. Indeed, Nizet (1951) reports that reticulocytes from the blood of protein-depleted dogs are incapable of transforming themselves into adult erythrocytes if they are incubated *in vitro*, contrary to reticulocytes from the blood of non-depleted dogs.

However, maturation of these cells becomes possible by adding a mixture of amino acids to the deficient blood.

In vivo the maturation block of the reticulocytes is suppressed by a refection diet with an adequate amount of proteins. That is why the reticulocyte crisis is followed by an increase in the number of adult erythrocytes at the same time as the reticulocytes disappear almost entirely from the circulating blood.

However, if the protein intake during the refection period is insufficient, or the institution of this diet too late, only the transformation of erythroblasts into reticulocytes becomes possible, the maturation of reticulocytes apparently being unable to take place: this hypothesis might explain the progressive increase in blood reticulocytes observed in rats, which become ever more anaemic during an unsuccessful attempt at restoration.

It is nevertheless true that certain peculiarities in the evolution of the protein-deficiency anaemia remain without explanation, even if one assumes two different maturation blocks, the first between erythroblasts and reticulocytes, the second between reticulocytes and mature erythrocytes.

Thus, it is difficult to understand why a protein-deprived diet induces reticulocytopenia only at the beginning; whereas at an advanced stage anaemia is, on the contrary, accompanied by an increase in the reticulocyte level (Aschkenasy, 1944–6). On the other hand, the diminution of the radioiron uptake which occurs at the early period of protein starvation is followed by a moderate augmentation of this uptake at a later stage (Bethard *et al.*, 1958). All these data indicate a paradoxical increase of erythropoiesis which may be the consequence of an abnormal haemolysis.

2. The Role of the Haemolysis

The possibility of an excessive destruction of erythrocytes in protein-depleted rats is suggested by several observations *in vitro* as well as *in vivo*.

(1) Erythrocytes of protein-depleted rats show an increase in their sensitivity *in vitro* to digitonin (Aschkenasy, Delmonte and Eyquem, 1956) (Table 1.1)

TABLE 1.1

Effect of Digitonin (final concentration of 2 μg/ml) on Erythrocytes of Normal and Protein-starved Rats

(Microscopic study. Means ± S.E.M.)

Percentage of protein in the diet	Number of rats	Percentages of haemolyzed erythrocytes after				
		5 min	15 min	30 min	45 min	60 min
18	6	16·2 ±3·7	27·6 ±3·5	38·1 ±4·2	44·3 ±5·9	49·2 ±5·9
0	6	70·3 ±4·4	81·2 ±4·2	86·1 ±4·3	88·9 ±4·0	91·1 ±4·1

and saponin (Delmonte, 1957; Aschkenasy, Blanpin and Coquelet, 1959) as well as a decrease in their mechanical (Table 1.2) and alkaline resistances (Delmonte, 1957).

TABLE 1.2

Effect of Mechanical Trauma (90 rotations/min during an hour) on Erythrocytes of Normal and Protein-starved Rats

(Microscopic study; means±S.E.M.). Number of rats in parentheses

Technique	Percentage of haemolyzed erythrocytes	
	Controls	Protein-starved rats
De-fibrinated blood in not siliconized glass flasks	31·1±2·9 (9)	52·1±5·0 (9)
Heparinized blood in siliconized glass flasks	17·7±3·0 (4)	27·6±3·1 (5)

(2) After their tagging with radio-chromium and intravenous injection into normal rats, erythrocytes from protein-depleted rats have a shorter life-span than erythrocytes from non-deficient rats (Aschkenasy, Delmonte, Guérin and Guérin, 1956) (Fig. 1.5).

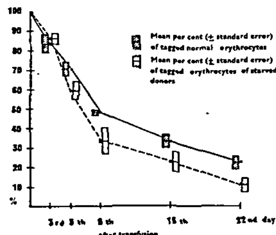


FIG. 1.5: APPARENT SURVIVAL TIME OF NORMAL ERYTHROCYTES (——) AND OF ERYTHROCYTES OF PROTEIN-DEPRIVED RATS (– – –) AFTER THEIR TRANSFUSION INTO NORMAL RATS

Ordinate—per cent of erythrocytes surviving.
Abscissa—days after transfusion.

(3) The excessive haemolysis produced by protein deprivation may be responsible for the accumulation of iron found in the spleen in this dietary status (Aschkenasy, 1944–6), although the lack of utilization of iron, due to the inhibition of haemoglobin synthesis, is also to be considered as a possible cause of this phenomenon (Ventura, 1959).

Haemolysis probably plays a role, not only in the moderate increase in blood reticulocyte levels observed at the end period of protein starvation and, in general, in all protracted protein poor diets (Orten and Orten, 1943), but also in the increasing reticulocytosis which accompanies the aggravation of anaemia during unsuccessful attempts at restoration.

3. Factors Responsible for the Maturation Block and the Haemolysis

Several arguments suggest that the maturation block and haemolysis are both caused, not merely by a lack of nitrogenous building materials of blood cells, but also by functional disturbances of the bone marrow, partly conditioned by hormonal imbalance, as well as by biochemical changes in the blood serum.

(a) *The Maturation Block*

This block, being a very early phenomenon, cannot be related to the exhaustion of protein reserve stores. It appears to be due rather to an enzymatic inhibition, which may be induced by a hormonal imbalance. It is noteworthy, indeed, that thyroidectomy accentuates, whereas thyroxine injections counteract, the erythroblastic as well as the reticulocytic block in protein-depleted rats (Aschkenasy and Pariente, 1952; Aschkenasy, 1954, 1960*b*). That is why haematopoietic utilization of radioiron is increased by this hormone and reduced, on the contrary, by thyroidectomy, as has been shown by Austoni *et al.* (1958) even in non-deficient rats.

It may be that thyroxine does not directly influence the cells of the bone marrow but acts merely by activating the oxidations in the bone marrow as well as in other tissues. Indeed, the fall of the reticulocyte levels at the beginning of protein deprivation coincides with a prompt decline in oxygen consumption, and both phenomena can be prevented by injections of thyroxine. It is noticeable that cortisone is also efficient on that account (Aschkenasy, 1960*b*). Conversely, the protein-depletion anaemia may perhaps be caused by a decrease in tissular oxidations following the involution of the thyroid.

However, it is also possible that the lack of proteins directly inhibits certain oxidizing enzymes and thereby decreases the tissular metabolism, the inactivation of the thyroid occurring only as a secondary phenomenon.

Should the medullary block characteristic of the deficiency anaemia result directly from the reduction of tissular oxidations, an artificial stimulation of the latter would reduce the anaemia of protein-depleted rats or, at least, induce a discharge of reticulocytes into the circulation. In order to ascertain the validity of this hypothesis, we injected, for one week, adult male rats, deprived of alimentary proteins, with 1 mg/ day of 2,4-dinitrophenol (DNP), which is a well-known stimulant of oxygen consumption. The evolution of the anaemia was compared with that observed in controls injected with physiological saline, during the same period of protein starvation (Aschkenasy, 1959*b*).

In rats deprived of proteins over a period of 50 days, DNP partly counteracted the aggravation of anaemia as it occurred in saline-injected control rats on the same diet. Indeed, whereas in the latter the average decrease in erythrocyte levels was of 1,470,000 per cu mm, and that in haemoglobin of 2·2 g, on the contrary, in DNP-treated rats the average decrease did not exceed 680,000 erythrocytes and 1·2 g haemoglobin.

On the other hand, the drug was inefficacious in rats deprived of proteins for 70 days. Moreover, no differences in blood reticulocytes have been observed between the controls and the DNP-injected rats.

Nevertheless, these experimental data suggest that protein-depletion anaemia is, to a certain extent, at least at its early stage, conditioned by the rate of tissular oxidations, even without any obligatory mediation of the thyroid gland. Indeed the tissular effect of DNP, although accompanied by an increase in the peripheral utilization of thyroxine, does not stimulate the secretion of this hormone (Goldberg, Wolff and Greep, 1955; Castor and Beierwaltes, 1956).

Besides the reduction of the tissular respiration, a disappearance of some humoral erythropoietic factor might also be considered as a cause of the erythroblastic block in the protein-deprivation anaemia.

Indeed, a polycythemic reaction can be induced, even in protein-deprived rats, by administration of cobalt (Orten and Orten, 1945) which seems to act through a release of erythropoietin (Goldwasser *et al.*, 1958).

(b) *The Haemolysis*

Contrary to the maturation block, haemolysis is a late phenomenon in the course of protein deprivation, and thus, it may be caused by a lack of nitrogenous materials leading to the production of abnormal red cells.

However, some experimental data suggest that the excessive haemolysis may be due to a disturbance of the fat metabolism, induced itself by protein deprivation. Indeed, erythrocytes of protein-starved rats can be protected partly *in vitro* against haemolysis produced by saponin and digitonin, as well as by alkalis or mechanical trauma, by the adding of normal rat serum to the erythrocyte suspension. On the contrary, serum of protein-depleted rats is non-effective, unless it is supplemented by lecithin and cholesterol or even only by lecithine (Delmonte, 1957).

On the other hand, the resistance *in vitro* to saponin of erythrocytes taken from protein-starved rats is improved if the latter receive a diet containing larger amounts of vegetable fat. In such a diet the blood serum shows higher cholesterol levels, whereas its content in proteins and phospholipids remains unchanged (Aschkenasy, Blanpin and Coquelet, 1959).

Thus, the insufficiency of protection shown by a "protein-depletion" serum seems to be at least partly conditioned by its low content in both cholesterol and phospholipids, this content being approximately of 50 to 70 mg and 35 to 50 mg per 100 ml, respectively, in protein-starved rats against 80 to 100 mg per 100 ml in normally nourished rats.

However, erythrocytes taken from protein-starved rats still exhibit *in vitro* a certain decrease in their resistance to various haemolytic agents, even after the adding of normal rat serum (Delmonte, 1957). It is, therefore, reasonable to assume that a structural defect of these cells is also a contributing cause of the haemolysis, but up until the present all investigations in this field have failed: indeed, in the first place, no differences have been found between the electrophoretic pattern and the resistance to alkalis of haemoglobins from protein-starved and from protein-fed rats. On the other hand, the proportions of total lipids, cholesterol and phospholipids are similar in the stroma of both varieties of erythrocytes (Aschkenasy, Blanpin and Coquelet, 1959), but it is not impossible that future investigations performed with improved methods (such as chromatographic analysis of stroma phospholipids) would lead to a different conclusion.

Up to date, no systematic research has been undertaken concerning the possible role of hormonal imbalance in the haemolysis of protein depletion. However, it has been shown that testosterone propionate, injected into protein-depleted rats, does not even slightly increase the resistance of erythrocytes to saponin *in vitro*, although it counteracts significantly the development of the anaemia (Blanpin and Aschkenasy, 1959).

B. Pathogenesis of the Protein-depletion Leucopenia

Leucopenia induced by the removal of alimentary proteins may be due *a priori* either to a maturation

block or to an intravascular lysis or, perhaps, even to a claustration of leucocytes in certain organs. On the other hand, endocrine imbalance surely plays an even more important role than in the pathogenesis of the anaemia.

The possibility of the retaining of leucocytes in the tissues is apparently to be excluded in regard to the eosinophils, as has been demonstrated by systematical enumeration of these cells performed on sections and imprints of spleen, lung, kidney, thymus and lymph nodes, as well as on sections of stomach and intestine (Aschkenasy and Jobard, 1960).

Intravascular lysis seems to be partly responsible, at least for the lymphocytopenia. Indeed, protein-starved rats show, in the blood, a considerable increase in the number of damaged lymphocytes (Aschkenasy, 1944–6).

Nevertheless, the principal cause of the quantitative decrease in lymphocytes, as well as in neutrophils and eosinophils, appears to be the arrest of their maturation in the bone marrow and in the lymphoid organs. Simultaneously with the reticulocyte crisis, one observes, at the beginning of a refection diet, a prompt increase in the blood level of all kinds of leucocytes. This phenomenon can only be explained by an accelerated maturation of these cells in the haematopoietic organs, followed by their delivery into the blood stream.

As previously shown, both the involution of thyroid and the relatively increased activity of the adrenal cortex may play a role in the blocking of the lymphocytopoiesis in protein malnutrition. The agranulocytosis also seems to be favoured by the hypothyroidism. On the other hand, eosinopenia has apparently no hormonal origin in protein starvation (Aschkenasy, 1955).

IX. GENERAL CONCLUSION

1. Production of blood cells and haemoglobin requires a continuous supply of proteins. If this supply is inadequate, the need for haematopoiesis tends to be satisfied by the utilization of tissue proteins even at the expense of other functions. This explains the late appearance, as well as the moderate degree, of the protein-depletion anaemia.

2. Such an anaemia is hypochromic and microcytic. It is accompanied by leucopenia, with an almost complete disappearance of blood eosinophils and an atrophy of lymphoid organs.

Two different mechanisms seem to be responsible for the anaemia: (1) a maturation block which appears a long time before the exhaustion of the protein reserve stores. It concerns, on the one hand, the transformation of erythroblasts into reticulocytes and, on the other hand, the transformation of reticulocytes into mature red cells; (2) an excessive haemolysis, which seems to be due to a defective structure of the red cells as well as to an inadequate protection exercised by the blood serum of the protein-depleted animals. That is because this serum contains insufficient amounts of certain anti-haemolytic factors such as phospholipids and cholesterol.

Both maturation block and haemolysis explain the high amount of erythroblasts in the bone marrow of protein-depleted rats, as well as the changes of the blood reticulocyte levels during the development of anaemia and the recovery period.

3. Like anaemia, the protein-depletion leucopenia is to be ascribed to a maturation block of the white cell precursors in the bone marrow and in the lymphoid organs, as well as to an abnormal destruction of these cells (at least the lymphocytes) in the blood vessels.

4. The haematopoietic potency of the alimentary proteins depends principally on their amino acid composition, and the inferiority of several vegetable proteins is related to the inadequate amount of certain essential amino acids such as lysine, but simultaneous feeding with different deficient proteins permits a reciprocal compensation of their deficiencies.

Almost all amino acids which are indispensable for growth are also necessary for haematopoiesis. The beneficial effects of any amino acid are apparent whenever the organism is deprived of the latter or has an exceptionally strong need for it in order to compensate an abnormal loss of this amino acid or to neutralize some toxic compound.

5. According to some experiments concerning the eosinopenic potency of casein and various amino acids, it appears that even the physiological variations of the white cell picture depend to a certain extent on the protein intake.

6. Protein depletion induces anaemia and leucopenia not only because it deprives the haematopoietic organs of the amino acid constituents of blood cells and haemoglobin, but also because it prevents the production of certain vitamins, either by inhibiting their microbiological synthesis in the digestive tract (folic acid, niacin) or by suppressing the supply of their metabolic precursors such as tryptophan for niacin.

7. On the other hand, a hormonal imbalance, particularly an inactivation of thyroid and testicles, and an hyperactivity of the adrenal cortex also play a role in the development of the blood disorders.

Indeed, the evolution of anaemia as well as of leucopenia can be considerably modified by the removal of thyroid, testicles or adrenals, as well as by the

administration of various hormones. Testosterone and thyroxine are particularly effective in the production of red cells. Thyroxine seems to act, at least partly, by increasing the tissular oxidations as is suggested by the anti-anaemic potency of dinitrophenol.

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The Effects of Malnutrition on the Eye: with Special Reference to Work with Experimental Animals

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Contents

I. INTRODUCTION*

It not uncommonly occurs that, where two areas of specialization in medicine overlap, the "common ground" to each tends to be neglected by workers from either side. Each feels a lack of competence concerning the other's subject, and is attracted by the more congenial problems strictly within the confines of his own discipline. However, when attention is devoted to one of these "no man's land" areas of research it may be expected to yield results of special interest to those on both sides, and may even bring fresh light to bear upon old problems in other fields, when they are considered from a new point of view. Thus, F. G. Hopkins, in his Linacre Lecture for 1938, said "It is true . . . to say that in scientific borderlands not only are facts gathered that are often new in kind, but it is in these regions that wholly new concepts arise."

Two such specialities are ophthalmology and nutrition, and there is a widely-scattered literature dealing with both clinical and experimental aspects of malnutrition and the eye. In view of the unique importance of the eye, and the profound effects which disturbed nutrition may have on the delicate balance between its various parts, it is surprising that so much attention has been paid to certain much less devastating results of malnutrition than to the subject now to be considered.

The eye is one of the most complex organs in the body, and has been studied in all its aspects of structure and function, both normal and pathological, to a degree commensurate with its complexity and its importance to the organism. More perhaps than any other organ, the eye demonstrates an almost uncanny organization and harmony between its various parts, and this, to the one end that, with the maximum efficiency, light rays may be absorbed, converted into electrical impulses and transmitted to the brain in such a way that a correct visual impression of the environment may be obtained. Disease of any one part of the eye will result in diminution of efficiency of the whole organ, and may even produce total loss of function as, for instance, in a dense central corneal leucoma.

The eye conditions which will be discussed here are clearly of primary importance in man, and although most of the evidence that will be presented has been derived from animal experiments, yet, because of the underlying implications for human disease, it has been thought best to begin by introducing the subject from the point of view of human pathology, before going on to review recent related experimental work.

It will not be profitable to review extensively the early literature on the subject, and reference will be made to this only with a specific purpose in mind. Several sources provide a good introduction to certain aspects of nutritional eye disease: the monograph of Jackson (1925) has a valuable chapter, and the reviews of Mayer (1920), Blegvad (1924) and, more recently, that of Keys and his collaborators in the Minnesota experiment (1950), should also be consulted in this connexion.

For the purpose of this review "malnutrition" has been taken to mean undernutrition resulting from a faulty diet. Overnutrititon might have been included, but so far as the eye, at least, is concerned, little is known about its effects, except in certain types of hypervitaminoses. These have been shown to produce congenital defects which are, paradoxically, similar to those which result from a deficiency of these same vitamins. The cataractogenic and other effects of galactose, naphthalene, thallium, and other toxic agents, although produced when these substances form part of the diet, are not considered to be nutritional conditions. Furthermore, metabolic disorders in which the eyes are affected, such as diabetes mellitus and hepato-lenticular degeneration, although, in a sense, nutritional in origin, are not due to a faulty diet.

II. HUMAN BACKGROUND

A. General Undernutrition and Starvation

It is well known that in states of extremely low caloric intake, due to enforced fasts, famines, or wasting diseases, the eye loses its normal lustre and recession of the globe takes place. However, the degree of undernutrition and starvation that is compatible with survival in man appears to have less effect on the eye than on most other organs. Within the eye, it would seem that the avascular lens is more susceptible than other ocular tissues. Bellows (1944) quotes Jess (1930) and Palich-Szántó (1924) for reports of cataract developing after such febrile illnesses as typhus, malaria, meningitis, smallpox, scarlet fever and encephalitis lethargica, as well as other instances attributed to long-continued lactation, severe nutritional disturbances, hunger oedema, and marked haemorrhage.

* The survey of the literature pertaining to this review was completed in February, 1959.

A case of bilateral sub-capsular cataract complicating anorexia nervosa was recently reported to the Ophthalmological Society of the United Kingdom (Miller, 1958). In all these instances it is impossible to establish a causal relationship between the inanition and the lens changes, and the evidence is only suggestive.

Most ocular lesions attributable to malnutrition observed in prisoners-of-war, and the civil population living under conditions of privation during the two world wars, seem to have been caused either by the lack of vitamin A or by deficiency of one or more members of the vitamin-B complex. However, a very interesting condition, named "superficial polymorphic keratopathy" by Djacos (1949), and reported by him in cases of famine oedema occurring during the second world war in Athens, appears to be quite different from the corneal changes due to vitamin deficiency, which will be described later, and merits further study. Under the slit-lamp microscope the corneal nerves were seen to be increased in thickness and surrounded by a shiny sleeve, or sometimes showed a granular appearance. Djacos thought that these changes were due to oedema, because they gave way to a normal appearance when the peripheral oedema disappeared. In addition, the pre-corneal film was irregular when stained with fluorescein and there was a diffuse increase in the permeability of the epithelium with round or oval sub-epithelial "elements," which never went deeper than Bowman's membrane. Also from Greece comes the less-detailed description of Petzetakis (1950) of eye changes in starvation. These consisted of oedema of the lids and conjunctiva, hypoaesthesia of the cornea, sluggish pupillary reflexes, diminution of lachrymal secretion, and irregularity and oedema of the superficial corneal layers.

The Minnesota experiment in human starvation (Keys *et al.*, 1950) revealed no ocular changes, apart from the interesting observation that the sclera and conjunctiva were unusually devoid of blood vessels, the whites of the eyes resembling unglazed porcelain and failing to redden even with soap solution. The general remarks on the care required in attempting to interpret eye conditions in malnourished communities, in the light of experimental results, from both man and animals (see Section H), are pertinent here.

B. Night Blindness, Xerophthalmia and Keratomalacia

The present purpose of the writer is merely to underline the importance of vitamin-A deficiency in man, and to call attention to certain aspects of the epidemiology of the condition. Standards texts should be consulted for clinical descriptions, etc.

A deficiency of vitamin A has been, and probably still is, the most important nutritional disorder affecting the eyes, resulting frequently in blindness. That the condition has been recognized and effective treatment known for it for thousands of years, is demonstrated by many references (as far back as the Ebers papyrus, written about 1600 B.C.) to the value of liver, the storehouse of the vitamin, for eye trouble.

The manifestations of vitamin-A deficiency tend to arise in their florid form in two groups of individuals:

(*a*) subjects of all ages during times of famine and war;

(*b*) very young children of malnourished communities.

(*a*) For examples of the first, we may take from the earlier literature the accounts of fasting during the Lent Quadragesima in Russia, resulting in keratomalacia (Blessig, 1866), the occurrence of night blindness and xerosis conjunctivae in Poland (Budzynski and Chelchowski, 1916); of keratomalacia in babies of the poorer classes reared on sweetened skimmed milk in Denmark before and during the first world war (Bloch, 1921, 1924*a*, 1924*b*); and keratomalacia in Chinese soldiers on poor rations (Pillat, 1929). More recently, in a Nazi concentration camp in Central Europe night blindness was universal, Bitôt's spots and xerosis corneae were frequent, but keratomalacia was seen only in young children (Salus, 1957). A final instance may be taken from the area where the writer is at present working. Central Tanganyika is subject to droughts, and during a resultant famine in 1953–4 Balletto (1954) found many cases of keratomalacia and corneal opacities in famine camps and a government school.

(*b*) There are four features of the ocular effects of vitamin-A deficiency in malnourished communities which should be noted:

(i) The highest incidence occurs in early childhood, from about the end of the first year until the third or fourth year of life.

(ii) It is in this "toddler" age group that the most devastating effects, namely xerophthalmia and keratomalacia, occur with their highest frequency.

(iii) The male is more susceptible than the female, and this is also true in experimental animals (*see* p. 43).

(iv) Ocular hypovitaminosis A is one of the most frequent concommitants of protein malnutrition or kwashiorkor (McLaren, 1958*a*).

Vitamin-A deficiency remains one of the most common nutritional diseases, despite all that is known about its aetiology. It was one of the subjects taken up at a recent conference on nutritional disease (McLaren, 1958*d*), and the need for action has been recognized by the Joint FAO/WHO Expert Committee on Nutrition (1958). It is particularly widespread in South-East Asia, but it is becoming increasingly evident that it often reaches serious proportions in those parts of Africa where the red

palm (*Eloeis guineensis*) does not grow, also in certain parts of Central and South America. In Java, and some of the other islands of Indonesia, the problem is staggering in its magnitude; one hospital alone has records of more than 11,000 cases blind from vitamin-A deficiency from 1934 to 1954. The association with kwashiorkor is particularly marked here, about three in four of all the children with xerophthalmia also having kwashiorkor (De Haas, Posthuma and Meulemans, 1940; Oomen, 1953; Darby and McLaren 1957). The same association, but to a lesser degree, is also found in many other places, including some parts of India (e.g. Achar, 1950; Khalap, 1956; Manchanda and Gupta, 1958), but the writer found (McLaren, 1956) that in Orissa the keratomalacia and kwashiorkor cases were two distinct groups.

The detection of the milder degrees of vitamin-A deficiency in the field presents a number of problems. Conjunctival and corneal changes are relatively late manifestations of the condition; the young child is the age group most commonly affected; plasma vitamin-A levels vary widely in health and do not accurately reflect vitamin-A status; and impairment of dark adaptation as a sign of early retinal dysfunction requires full co-operation. In view of all these difficulties it may be that the electroretinogram, which has been shown to be reduced in amplitude in man in vitamin-A deficiency (Bornschein and Vukovich, 1953) will prove to be helpful. Dhanda (1956) has reported from India that the electroretinogram is completely extinguished in young children with night blindness due to vitamin-A deficiency, and that it reappears as the night blindness improves under treatment.

In concluding these introductory remarks on the subject of vitamin-A deficiency, which are intended to sketch in the human background for later detailed consideration of experimental aspects of the subject, it might be as well to point out that disease states in man are frequently of complex aetiology, and this is perhaps especially true of nutritional disorders. It has been suggested (Yap, 1956) that xerophthalmia and keratomalacia are different, if related, conditions, and that the latter is primarily due to protein deficiency. The writer has elsewhere (McLaren, 1959*a*) criticized this evidence, and a study of this problem in experimental animals is described later (*see* p. 42). Furthermore, the possibility that a nutritional deficiency may be associated with, enhance the effect of, or even be attributable to, a parasitic infection has to be borne in mind. For instance, recently, the role of *Onchocerca volvulus* in the causation of both the anterior and posterior segment eye lesions frequently associated with onchocerciasis has been called into question (Choyce, 1958; Kershaw, 1958), and there is some evidence that vitamin-A deficiency may play a part in the production of retinal damage (Rodger, 1957).

C. Deficiency of Vitamins of the B Complex

The present state of knowledge of human nutrition is not sufficiently advanced to deal with the vitamins of this group separately. Many of them occur together in foodstuffs, and human dietaries tend to be deficient in several of these factors at once, and consequently isolated deficiency states in man are rare.

The symptomatology of vitamin-B complex deficiency is extremely diverse, and while beriberi, pellagra and the oro-genital syndrome are fairly clear-cut conditions, primarily due to deficiency of thiamine, nicotinic acid, and riboflavin respectively, there are also other less well-defined conditions the precise aetiology of which is still not clear, although there is good evidence that a deficiency of members of the B complex is involved. These include nutritional amblyopia, corneal epithelial dystrophy, nutritional macrocytic anaemia, Wernicke's encephalopathy, the burning-feet syndrome, and certain neurological conditions including a peripheral neuritis and spastic paraplegia.

1. Nutritional Amblyopia

Diminution of visual acuity in malnourished communities has been known to occur for a long time, and ocular symptoms have occasionally been reported in pellagra and in beriberi. Largely due to the prominence and interpretation given to the early Japanese literature by Elliott (1920) there has grown up a belief that ocular involvement is frequent in beriberi. However, the extensive experience in Japanese prison camps during the second world war, of which the excellent accounts of Smith and Woodruff (1951) and Denny-Brown (1947) are particularly good examples, does not support an association with beriberi but does suggest that deficiency of some other member of the vitamin-B complex, possibly riboflavin, is responsible. The critical review of the early Japanese literature by Denny-Brown (1947) has shown that, if anything, the evidence is against, rather than for, an association of the eye symptoms with beriberi.

It is unnecessary here to go into the clinical aspects of the condition but its widespread occurrence and disputed aetiology should be noted. As stated, the consensus of opinion from the study of the condition in prison camps was that riboflavin, or a closely related member of the B complex was involved. The conditions described by Moore (1930) in West Africa, Métivier (1941) in Trinidad, and Landor and Pallister (1935) in Malaya, appear to be identical with one another and with the optic nerve lesions in prisoners, and all responded to treatment with rich sources of the

B vitamins. The condition known in Japan as "Shibi-Gattchaki" disease was initially attributed by Irinoda and Sato (1954) to riboflavin deficiency. In addition to skin and mucous membrane lesions the optic nerve was described as being frequently involved. The most recent accounts come from Jamaica, where Cruickshank (1956) has reported one hundred cases of a "neuropathic syndrome of uncertain origin" in which amblyopia was a prominent feature. Dietary insufficiency was suspected, but no clear evidence was obtained and, unfortunately, response to therapy does not appear to have been studied. Degazon (1956), on the same island, found that in a group of 298 cases of amblyopia, without other neurological signs, early cases were benefited by vitamin-B complex therapy, but postulated a vasodilator rather than nutritional effect of these substances.

Tobacco-alcohol amblyopia, which is very similar in symptomatology, has been reported (Carroll, 1944) to respond to thiamine, or to a greater degree to the whole vitamin-B complex, even while consumption of the toxins is continued. A similar claim has recently been made for vitamin B_{12} (Heaton, McCormick and Freeman, 1958). In this connexion it may be recalled that the optic nerves are frequently involved in pernicious anaemia and sub-acute combined degeneration of the cord, and further work may show that either folic acid or vitamin B_{12}, or even some as yet unknown factor related to the B complex, may be curative in the nutritional amblyopia of the tropics.

2. Corneal Epithelial Dystrophy

This name was given by Métivier (1941) to a condition in which the corneal epithelium was superficially eroded in a punctate fashion, sometimes in the form of a series of fine dots running across the cornea at the lowest part of the pupillary area. A similar condition was seen in the Japanese prisoner-of-war camps (Smith and Woodruff, 1951), usually associated with amblyopia and other conditions responding to vitamins of the B complex. Another excellent account of what appears to be the same condition comes from the Philippines (Nanagas, 1953). Although good results have been claimed with B-complex vitamins in these cases, and riboflavin, in particular, has been found effective, nothing comparable has ever been described in experimental animals suffering from deficiency of any of the B-group vitamins.

3. *Corneal Vascularization*

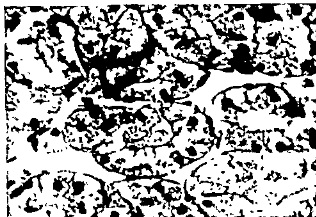
The growth of capillaries from the limbic plexus into the normally avascular cornea, as the result of nutritional deficiency in man, was first described by Sydenstricker *et al.* (1940) and attributed by them to riboflavin deficiency. Most of their cases also had photophobia, dimness of vision, conjunctivitis, and circum-corneal injection. There followed numerous confirmatory reports, some suggesting that there was a high incidence of corneal vascularization in otherwise healthy people in North America and Europe. It soon became evident that errors had arisen because many investigators were unfamiliar with the physiological variations of the limbic plexus. It is now generally recognized that corneal vascularization due to riboflavin deficiency occurs in man, as it does in animals, only in marked deficiency, accompanied by such ocular symptoms as have been mentioned above, and often by symptoms in other parts of the body attributable to deficiency of members of the B complex. As yet, riboflavin alone of the B vitamins has been shown to be capable of reversing the condition in man. The total absence of conjunctival and corneal changes in a prolonged human riboflavin-deprivation experiment is discussed in Section H (p. 34).

4. Angular Blepharo-conjunctivitis

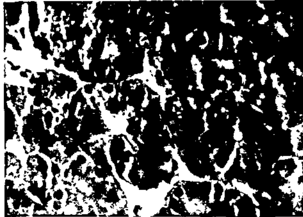
Fissuring of the lids at the outer canthus is frequently associated with similar angular stomal lesions and other manifestations which usually respond to therapy with B-complex vitamins. Thus, angular blepharo-conjunctivitis is a prominent feature of "Shibi-Gattchaki" disease. This is a condition occurring in certain parts of Japan, in which a variety of lesions affecting the conjunctiva, cornea, iris, optic nerve and retina are reported to respond to treatment with riboflavin and nicotinic acid (Irinoda and Yamada, 1956). It would seem that Irinoda has revised his opinion with regard to the aetiology of this particular sign at least, for in a recent paper (Irinoda and Mikami, 1958) both clinical and experimental evidence is brought forward to support the contention that pyridoxine deficiency is responsible. This will be discussed further later (p. 45). In certain parts of the world, angular blepharo-conjunctivitis and other skin and mucous membrane lesions accompany kwashiorkor with some frequency (McLaren, 1958*a*).

D. Discrete Colliquative Keratopathy (D.C.K.)

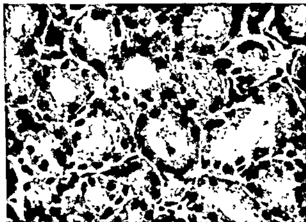
This name has been chosen to describe a condition to which attention was first called by Blumenthal (1950, 1954) in the South African Bantu. It has also been identified more recently in East Africa (McLaren, 1958*b*) and in West Africa (Rodger, 1958) but has so far not been reported outside the African continent. As the name suggests, it consists of a clean perforation of the cornea, usually in the lower half not far from the limbus, in an otherwise apparently "quiet" eye. In the complete lesion, iris is drawn into the wound and the final result is healing with scar formation resulting in leucoma adherens. D.C.K. has been con-



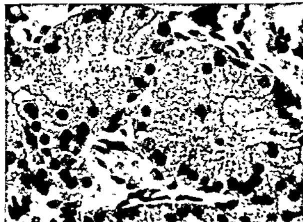
(*a*) HARDERIAN GLAND OF A PROTEIN-DEFICIENT PIG
Stained with haematoxylin and eosin (×400).



(*b*) HARDERIAN GLAND OF A NORMAL PIG FOR COMPARISON WITH (*a*)
Same stain and magnification as (*a*).



(*c*) HARDERIAN GLAND OF A RAT FED ON CASSAVA
Stained with haematoxylin and eosin (×400).



(*d*) HARDERIAN GLAND OF A NORMAL RAT FOR COMPARISON WITH (*c*)
Same stain and magnification as (*c*).

(*e*) HARDERIAN GLAND OF A RAT FED ON A PROTEIN-FREE DIET
Stained with haematoxylin and eosin (×400)

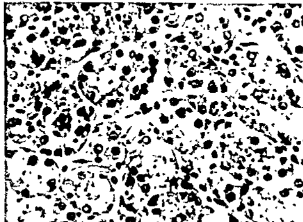


PLATE I



(*a*) Lids of a Normal New-born Rat

The zone where the lids will later separate and the inner surface of the lids, stain strongly with P A S positive. Stained with periodic acid-Schiff reagent and gallocyanin (× 145)



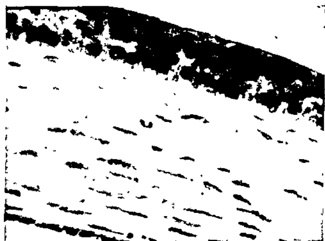
(*b*) Lids of New-born Rat from a Protein-deficient Dam

The zone of separation of the lids and the inner surface of the lids are devoid of P.A.S.-positive material. Same stain and magnification as (*a*).



(*c*) Corneal Epithelium and Superficial Stroma of a Rat Fed on Cassava

The epithelium consists of only two layers of cells, and the cells of the basal layer are swollen, with dense nuclei Stained with haematoxylin and eosin (× 375)



(*d*) Normal Rat Cornea

Same stain and magnification as (*c*), with which it should be compared.

Plate II



(*a*) CATARACT IN A PROTEIN-DEFICIENT PIG
The triradiate suture is well shown.



(*b*) SECTION OF THE SAME LENS AS (*a*).
The fibres are completely disorganized, many have the appearance of "bladder" cells, and cellular debris lies in the inter-fibrillar clefts. Stained with haematoxylin and eosin (×95).



(*c*) NORMAL PIG LENS
Same stain and magnification as (*b*).

(*d*) EYE OF NEW-BORN RAT FROM PROTEIN-DEFICIENT DAM
The lens is grossly disorganized. Stained with haematoxylin and eosin (×54)

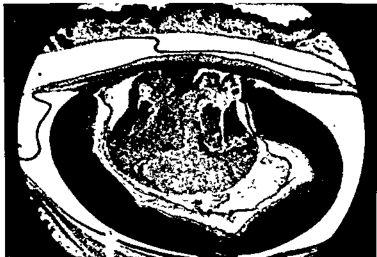


PLATE III



(*a*) Rat Cornea
Localized Hyperplasia of the Endothelium
Stained with haematoxylin and eosin (×400).



(*b*) Rat Cornea
Sub-epithelial Infiltration of Mononuclear Cells
Stained with haematoxylin and eosin (×960).



(*c*) "Spectacle Eye" in a Vitamin-A-deficient Rat



(*d*) Porphyrin Incrustation of the Lids in a Vitamin-A-deficient Rat

Plate IV

fused with, but is quite different from, keratomalacia (McLaren, 1959*c*), and although it is frequently seen in malnourished children, the writer has observed it in well-nourished children and adults, and any aetiological association with malnutrition is at present purely speculative. Nothing resembling D.C.K. has been seen by the writer in malnourished experimental animals, nor has it been described by others. Personal observations, with the slit-lamp microscope, of a preliminary nature (McLaren, 1958*c*) show that the lesion is primarily in the corneal endothelium.

E. The Lachrymal Glands

Little attention has been paid to the lachrymal glands in connexion with human malnutrition. In the first monograph written on kwashiorkor, Purcell (1939), from the Gold Coast, noted enlargement of the lachrymal and parotid glands in malnourished individuals. It is pointed out by Trowell, Davies and Dean (1954) that the lachrymal glands, like the parotids, appear, on direct inspection, to be enlarged in many African adolescents and adults. They also show a section (Fig. 17 of their monograph) of the lachrymal gland from a child aged three years, dying from kwashiorkor, in which there was atrophy of the acinar secretory cells with the cytoplasm shrinking down around the nuclei.

It is difficult to reconcile the apparent clinical prominence and the histological shrinkage of the lachrymal gland, and in any case, according to Adler (1953), the lachrymal gland is especially easy to expose in negroes, and this is because the eyes tend to be rather prominent. Furthermore, it is evident from the description of the normal histology of the lachrymal gland that its appearance varies considerably at different ages. Mann (1949) states, "The gland is small at birth, growing during the first few years after birth, with change in its character, the lumina increasing in size and the intervening connective tissue stroma becoming much thicker." Evidence that marked changes in the para-ocular glands can be produced by protein deficiency in experimental animals will be presented later (p. 38).

F. Cataract

Various nutritional deficiencies may result experimentally in cataract, and this subject is dealt with on p. 39. In man, however, there is, as yet, no clear evidence that malnutrition causes damage to the lens. The uncertain relationship between states of inanition and the development of lens changes has already been referred to (p. 30). The data compiled by Bellows (1944), Sorsby (1950) and others on the incidence of cataract in different countries are acknowledged by these writers to be very incomplete, open to many criticisms, and, in some cases, quite probably totally misleading. Despite the absence of reliable data, there is a widespread belief, based upon the long experience of numerous ophthalmologists in the tropics, that there is a higher incidence of cataract in the people of warm climates than in Europeans, also that cataract tends to occur there at an earlier age than it does in the West. India is outstanding in this respect, and it has been computed that approximately 100,000 cataract operations are performed annually in that country, but that five times this number would need to be done to deal with every case. In view of the paucity of factual information on both the incidence in the general population and the age of onset of so-called "senile" cataract it seemed to be worthwhile to mention some work on both these points from an area of India where the writer worked for five years.

Surveys of three large villages in the Khond Hills, Orissa, carried out between 1951 and 1953 (McLaren, 1955), in which almost all of the inhabitants were examined, showed that among 2,126 people of all ages there were 8 with mature cataract. All of these had sufficient impairment of vision for them to have been included on the register of the blind if they had been in England and Wales. According to Sorsby (1950), the number of registered blind in England and Wales in 1948 was 77,390, of whom 24·6 per cent, or 19,347, were blind due to cataract. This was roughly 1/44,400 of the total population. It is obvious that the incidence of cataract was very much higher in the Khond Hills villages than in England and Wales, even without taking into account the fact that the proportion of people over the age of 45 was nearly three times greater in England and Wales than in the Indian villages surveyed.

I am indebted to my former colleague, Dr. S. F. Thomas, of the Moorshead Memorial Hospital, for the details of 517 consecutive cases of cataract coming for operation. Table 2.1 shows the age incidence of the condition in the Khond Hills as compared with data taken from Sorsby (1950) for England and Wales.

In the Indian series juvenile cataracts accounted for the few cases under the age of thirty, and so-called presenile or senile cataract entirely for those above this age. Diabetic cataract was not seen, and the various forms of secondary cataract were not included. In the data for England and Wales, all kinds of cataract except congenital are included.* It is evident that a much higher proportion of all cataract cases occurs in the under-60 age groups in India as compared with England and Wales.

* The two sets of data are not identical in nature, nor could they be from such diverse sources, but with this proviso a comparison may tentatively be made.

TABLE 2.1

A Comparison of the Incidence of Cataract in the Khond Hills, India, and in England and Wales

1	2	3	4	5	6	7	8	9	10
Age in years	No. of cataracts in Khond Hills series	% in each group	% distribution of Khond Hills population	Relative % incidence of cataract (i.e. 3:4 as %)	No. of cataracts in Sorsby's data	% in each group	% distribution of England and Wales population	Relative % incidence of cataract (i.e. 7:8 as %)	Relative % incidence compared (i.e. 5:9)†
Under 30	7	1·4	67·5	0·14	—	—	41·0	—	—
30–39	16	3·1	14·2	1·14	4	0·1	14·6	0·10	11 4
40–49	73	14·1	8·2	8 95	51	1·2	14·8	1·32	6 8
50–59	193	37·3	3·6	54·26	218	5·2	13·1	6·44	8·4
60 & over	228	44·1	6·5	35·51	3931	93·5	16·5	92·14	0 4
Total	517	100	100	100	4204	100	100	100	

† *There is a much higher proportion of cataract in the Khond Hills in the three ten-year periods, leaving relatively few in the 60 and over group as compared with England and Wales.*

Further consideration of the possible relationship between malnutrition and cataract must be left over to the experimental section (p. 39), when considerable evidence will be brought forward to show that, in animals at least, such a relationship does exist.

G. Myopia

One raises, with some diffidence, the possibility of myopia being related to dietary deficiency, as the theorizings of Knapp (1939, 1943) concerning vitamin-D deficiency and "scleral rickets" are apt to come to mind and prejudice a consideration of this subject. Interest has, quite recently, been renewed in the inter-relationship of body growth and changes in the refractive state of the eye, in both man and experimental animals, by the work of Gardiner. He found (1954) that myopic London school children were growing more rapidly than others, and that the progress of the myopia was related to the rate of their growth (1955). He went on to examine their likes and dislikes with regard to diet, and came to the conclusion (1956) that they refused more items of rich sources of animal protein than other children. In his latest contribution (1958), Gardiner claims that the deterioration of myopia can be retarded by increasing the amount of animal protein consumed. Reference will be made later (p. 38) to his experimental work. One would only add here, by way of comment, that, from personal experience, refraction, using the electric retinoscope, is not a very precise method of examination, and the decision as to whether or not small changes have occurred may be easily influenced subjectively, unless the experimental conditions are strictly controlled.

A recent monograph entitled "Refraction and body growth," by Pendse (1954), consists of a study in young Indians in Poona. Although it does not answer the question as to whether or not human malnutrition has an effect on the refractive state of the eye, it does show, among other things, that there is a distinct difference between two racial groups examined. There was a highly significant difference between the refraction of young Brahmins and young "untouchables," the latter being more myopic, but whether this was genetically determined or due to environment it was not possible to say.

H. The Part of Local Irritation and Injury

The conjunctiva and cornea are the only transparent epithelial structures that are exposed to the environment. In this way, some form of local irritation would seem to be responsible for the predeliction of such conditions as Bitôt's spot, pinguecula, pterygium, diffuse conjunctival pigmentation and xerosis for the inter-palpebral fissure. Exposure of conjunctiva and cornea in this situation is much more prolonged than in the parts normally covered by the lids.

With regard to Bitôt's spots, there are numerous references to the part which exposure is thought to play in their development. A recent account is that of Salus (1957) who found that, in a German prisoner-of-war camp, Bitôt's spots had an epidemic-like onset

in the spring and, in the absence of a change of diet, he attributed this to exposure to solar radiation. He was also emphatically of the opinion that both xerosis conjunctivae and a secondary growth of xerosis bacilli were essential for a typical Bitôt's spot to develop.

Two further points suggest that these spots have anything but a simple nutritional aetiology. There have been many reports that they do not always respond to adequate vitamin-A therapy (Berliner, 1949), and they may occur in individuals with normal plasma vitamin-A levels (e.g. Roels, Debeir and Trout, 1958). The latter workers do not give individual values, but from the very large standard deviations in their Table VI it is evident that many of the values given for their Bitôt's spot cases must have been well within the accepted normal range.

The reaction of connective tissue to trauma is known to be abnormal in states of undernutrition (Platt, 1953), and it is possible that pinguecula and pterygium, which are degenerations of connective tissue occurring frequently in the tropics, may have a nutritional factor in their causation.

Perhaps the most striking demonstration of the conflicting results which may be obtained under natural and experimental conditions is the failure of any of the mental patients who took part in a controlled study to show any changes in the limbal blood vessels or corneal vascularization after as long as fifteen months on a diet severely restricted in riboflavin (Gordon and Vail, 1950). On the other hand, there is good evidence that riboflavin deficiency may be responsible for corneal vascularization in man under natural conditions (*see* p. 32). The finding of an increase in the threshold for flicker fusion (Horwitt *et al.*, 1949) in the same group of subjects does suggest that, as with vitamin-A deficiency, the retina may reveal impairment of function long before the anterior segment shows any signs.

I. The Possibility of Congenital Malformations

In Part III of this review, much will need to be said about the ocular defects which have been produced in the young of experimental animals by maternal dietary deficiencies of various kinds. The question naturally arises as to whether or not human malnutrition can have the same effect. The opinions of two authorities in this field, expressed in two recent reviews of the subject (Hogan, 1953, and Warkany, 1958), are in agreement that satisfactory evidence is not available for man, and that a causal connexion between nutrition and congenital defect is extremely hard to prove. Warkany points out that it is difficult to think of human reproduction under conditions comparable with the experiments resulting in congenital malformations. Amenorrhoea and sterility occur early in women who are undernourished and those who conceive are likely to miscarry or give birth to stillborn, premature or non-viable children. In contrast to the rat, the organogenetic period in which most malformations are determined is relatively short in man. There is, then, at present, no evidence that human malnutrition does cause congenital malformations, and for certain reasons it is unlikely to do so, but this does not deny the possibility of such a relationship being demonstrated in the future.

III. WORK WITH EXPERIMENTAL ANIMALS

A. General Inanition

Little attention has been paid to the effect of total inanition on the growth and function of the eyes since the classical work of Jackson and his associates, which was going on at the time when the concept of specific nutrient deficiency states was emerging. His earlier work was incorporated in a monograph (Jackson, 1925) and there were several subsequent papers on the same subject (Jackson and Smith, 1931; Jackson, 1932; McLennan and Jackson, 1933). This work, so far as the eye was concerned, showed that when the rat was subjected to total inanition the eye continued to increase in weight, frequently to a greater extent than any other organ. Similar results were obtained by Barry (1920) working with the young of rats whose mothers had been malnourished during pregnancy. McMeekan (1940) found that the weight of the eyeball of pigs on various diets was related to the age of the animal rather than to its body weight or state of nutrition, which is just another way of saying that the eye goes on growing, relatively little affected by the inanition.

Histologically the eye was found to present the normal structure in underfeeding with progressive histological differentiation, especially in the retina (Jackson, 1932). From the early literature Jackson cited accounts of histological changes in the retina of the fasting dog (Bich, 1895; Lodato, 1898*a* and 1898*b*). The latter reported finding "atrophic" changes in the retina, especially in the ganglion cell layer, and in the iris and choroid. Of special interest, in view of the importance of the endothelium of the cornea (p. 42) was a reference to "a partial loss of epithelium behind Descemet's membrane."

Finally, thirst has been reported to produce cataract in both rats (Kudo, 1921) and frogs (Durig, 1901).

B. Deficiency of Protein or Amino Acids

The decision has been taken to combine, in one section, the effects of general protein lack and those of specific amino-acid deficiencies. So far as the effect on growth is concerned, it would seem to be quite clear that lack of any one of the essential amino acids blocks protein anabolism and is tantamount to protein deficiency. It is, of course, true that certain of the essential amino acids have special functions over and above being involved in protein anabolism; for instance, the conversion of tryptophan to nicotinic acid, and the part of methionine in trans-methylation. However, the evidence that there is, in relation to the eye, will be seen to justify the present point of view. This conclusion was reached, even in the case of tryptophan, which was the first case of a deficiency of an essential amino acid shown to cause cataract, by the careful study of Schaeffer and Murray (1950) to which reference will be made later (p. 39).

1. Growth of the Eyeball

The work of Jackson and his colleagues has been referred to already in connexion with the effect of general inanition on the growth of the eye (p. 35). Here, it may be added that one of his later publications (Limson and Jackson, 1932) showed that, in protein deficiency also, the eye continued to increase in weight.

Addis, Poo and Lew (1936) measured, in albino rats, the quantities of protein lost by the various organs and tissues of the body during a fast, and found that the only organs from which there was no loss of protein were the eyes, the testicles, and the adrenals.

Lafon (1939) weaned albino rats on to diets deficient in either lysine or cystine. After varying periods on the diet they were killed, and the fresh and dry weights of various organs, including the eye, were compared with those of body-weight controls. The eye, kidney and testicle appeared to go on growing "at the expense" of other organs. In both groups, the percentage dry weight of the eyes was greater in the animals on the deficient diet than in the corresponding body-weight controls. Similar results in the offspring were obtained when a maternal deficiency of the two amino acids was studied. There was no significant difference between the effects of the two deficient diets, and the conclusion was drawn that each plays a passive role as material for protein synthesis. In view of the non-essential role of cystine it is strange that there was no difference, although it is known that the dietary balance between methionine and cystine is also of importance.

The writer has studied the growth of the eyeball of the rat in protein deficiency lasting from birth up to nearly two years of age (McLaren, 1958*d*). Figs. 2.1, 2.2 and 2.3 summarize the effects of three low-protein diets on both the fresh and dry weight of the eye. Diet 1 contained 4 per cent protein of good quality, diet 2 had 10·8 per cent protein, except during gestation and lactation, when it was increased to 17·6 per cent, and diet 3 consisted of cassava, containing only about 1 per cent protein, and was poor in all the essential amino acids.

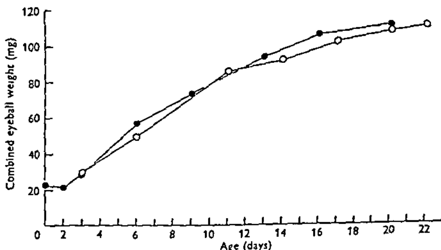


Fig. 2.1. Growth of the Eyeball, Before Weaning, in Rats from a Colony Fed on a Normal Diet, ●——●, and from a Colony Fed on a Low-protein Diet plus Pre-weaning Supplement, ○——○

(Courtesy British Journal of Nutrition)

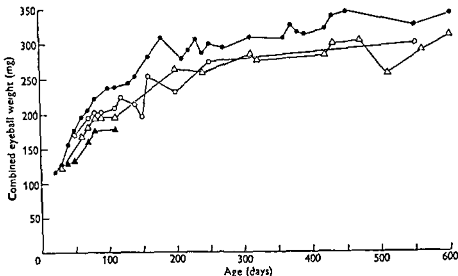


Fig. 2.2. Growth of the Eyeball, After Weaning, in Rats Fed on a Normal Diet, and on Low-protein Diets

(Courtesy *British Journal of Nutrition*)

●——●, normal diet; △——△, diet 1; ○——○, diet 2; ▲——▲, diet 3.

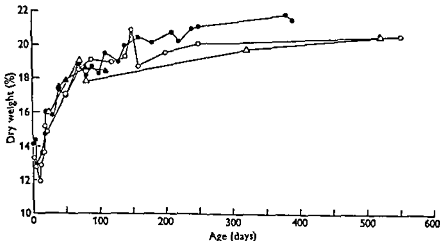


Fig. 2.3. Dry Weight of the Eyeball of Rats Fed on a Normal Diet and on Low-protein Diets

(Courtesy *British Journal of Nutrition*)

●——●, normal diet; △——△, diet 1; ○——○, diet 2; ▲——▲, diet 3.

The results may be summarized as follows: Fig. 2.1 shows the rapid increase in weight of the eye, which takes place from just over 20 mg for the combined eyeball weight at birth, to more than five times this figure at weaning. Before weaning, the eyes of the low protein colony rats fed diet 2 grew as well as those of the controls, reflecting the effect of the special supplementation, but afterwards they were consistently lighter. That the level of protein alone cannot account for differences in growth is shown by the almost identical curves for rats on diets 1 and 2 in Fig. 2.2, although the protein contents were 4 and 10·8 per cent respectively, with the latter especially deficient in lysine. The resistance of the eye to the deleterious

effects of gross quantitative and qualitative deficiency of protein is shown by the continued increase in the weight of the eye of rats on diet 3, despite a slow but steady loss of body-weight from weaning. Indeed the values for these rats are only about 25 per cent less than those for age-controls.

Fig. 2.3 shows that, in protein deficiency, the water content of the eye undergoes the same changes with age as those seen in control animals. This was even true for the sharp increase in water content of the eye from the 5th to 15th day of life, demonstrated first in normal rats by Donaldson and King (1936), who suggested that it might be related to the phenomenon of the opening of the eyes which takes place about this time. The failure of even severe protein deficiency to cause any deviation from the normal in respect of water content of the eye shows that the suggestion of Jackson (1915), which he made on the basis of the data of Lowrey (1913), that the high water content of the eye might explain its continued growth by increase in water content alone in states of inanition, is not well-founded.

An unusual and interesting approach to the study of the growth of organs has been made by Elchlepp (1956) using the chick eye. Evans blue, when injected, is normally restricted to the intravascular channels, but was found to appear in extravascular tissue in sites corresponding to areas of increased water solubility of ground-substance components. There was increased localization of dye in the orbital connective tissue during phases of accelerated growth of the embryo eyeball, and it is suggested that the state of the ground substance may determine the growth of the eye and other organs.

Finally, reference might be made here to the changes in the refractive state of the eyes of rabbits, which Gardiner and Macdonald (1957) attributed to a low protein diet. Although it is not known how the changes in refraction were brought about, it is possible that interference with growth was responsible. Animals in the experimental group received the diet which contained about 5 per cent protein for 2-, 4- or 6-week periods, after which they were given the stock diet. Two main conclusions were drawn: first, that the most striking refractive changes occurred after the resumption of the normal diet; and secondly, that the extent of the change was related to the length of time on the low protein diet. Repetition of this work is obviously desirable. The number of animals was very small to begin with, and was further reduced by deaths. Most of the animals contracted coccidiosis, and as one rabbit died from this, the others were all to some extent sick animals. Furthermore, these few animals showed a wide variation in refraction, and the examination itself is anything but a precise one.

2. The Ocular Glands and the Lids

Before the writer went to work at the Human Nutrition Research Unit of the Medical Research Council, London, Balfour (personal communication) had been studying for some years the changes in the salivary glands and pancreas produced by feeding rats on cassava. They were remarkably like those which have been described in these organs in kwashiorkor. It was decided to examine the Harderian, and the two lachrymal glands, of rats fed cassava and other low protein diets, and the study was extended to the lachrymal and Harderian glands of the pig in protein deficiency. The results have been published in full (McLaren, 1959*b*) and will only be summarized here. Of the para-ocular glands the Harderian was the most readily affected. In the pig, a diet containing 4·5 per cent protein resulted in the shrinkage and loss of cell substance shown in Plate I (*a*) after about 1 year of deficiency. In the rat (Plate I (*c*), (*d*) and (*e*)) the glands were normal for periods of up to two years in animals receiving a diet containing 4 per cent protein since weaning. In rats whose only source of protein was cassava, glandular atrophy occurred, with acinar cell shrinkage and round cell infiltration (Plate I (*c*)). Chemosis and incrustation of the lids with porphyrin secretion were present in many of the rats on the cassava diet in the terminal stage. The secretion was strictly limited to the lids and the end of the nose, and not nearly so widespread as in the usual description of "blood-caked whiskers."

Rats weaned on to a low protein diet, and then, at 50 days of age, given a diet entirely devoid of protein, lived for a month or two longer, and in the terminal stages showed marked chemosis with sticking together of the lids, but no porphyrin secretion. In the lachrymal and parotid glands there was shrinking down of the cytoplasm of the acinar cells, with atrophy of the whole gland, and early infiltration with round cells. In the Harderian gland these changes were much more advanced, with degeneration of the acinar cells and the acini filled with granular debris (Plate I (*e*)).

The lids of the rat are normally closed at birth, and the zone where separation is later to take place is at this stage filled with material which stains strongly with periodic acid–Schiff reagent (McLaren, 1957*a*), (Plate II (*a*)).

This material, probably mucopolysaccharide in nature, slowly disappears from this zone, which is free from it by the time the lids separate about the 15th day of life. The separation of the lids of the young of rats fed a diet deficient in protein was delayed and the P.A.S.-positive material was absent at birth (Plate II (*b*)). It was noted as long ago as 1895, by Von Bechterew, that there was a delay in the opening of the eyes of malnourished newborn puppies and kittens.

Mucopolysaccharide would seem to have an important role in this phenomenon, as it is known to have in certain other areas of increased tissue activity.

3. The Conjunctiva and the Cornea

Totter and Day (1942) were the first to describe corneal vascularization due to deficiency of an amino acid; in this case, of tryptophan, and of lysine. Subsequently, in deficiency of each of the other essential amino acids, corneal vascularization has been observed: leucine (Maun, Cahill and Davis, 1945), methionine (Sydenstricker *et al.*, 1946) and valine, histidine, isoleucine, phenylalanine, arginine and threonine (Sydenstricker, Schmidt and Hall, 1947). It has been stated that the depth of the invading vessels varies in different amino-acid deficiencies, for instance, being deep in valine deficiency, and superficial in tryptophan deficiency, according to Ferraro, Roizin and Givner (1947). These workers also describe a corneal dystrophy in their valine-deficient rats, consisting of keratinization of the epithelium with xerosis and keratitis. The impression one gains from the literature is that while vascularization of the cornea is common to all essential amino-acid deficiencies in the rat, the form which it takes, and the accompanying changes in the cornea, may vary considerably.

Diets deficient in, or devoid of, protein have also produced corneal vascularization in the rat (Sydenstricker *et al.*, 1946). Kaunitz *et al.* (1954), in a series of experiments in which many dietary factors were varied, claimed to have produced vascularization in rats on a 5 per cent casein diet. However, rats kept, by the writer, for two years on a diet containing 4 per cent casein, all failed to show vascularization. New growth of vessels into the cornea did result in rats fed cassava, and in rats fed a diet entirely devoid of protein from 50 days of age, as was found by Sydenstricker *et al.* (1946), and it was also shown that, the lower the level of protein in the diet before that time, the earlier the onset of vascularization (McLaren, 1959*b*).

In addition to vascularization, the corneal epithelium underwent a particularly interesting change in the animals fed on cassava. As shown in Plate II (*c*) it was greatly reduced in thickness, consisting of only two or three layers instead of the usual five or six. It would seem that this was due to an arrest in the division of the basal cell layer, for unlike in vitamin-A deficiency, where the basal cells remain unaffected, this layer was markedly abnormal. The basal cells were swollen with large clear cytoplasm and pyknotic nuclei. The cells appeared oedematous rather than "empty," and in this respect were like the basal cells seen near injuries to the cornea (p. 40). These changes resemble those seen in the human condition known as bullous keratitis.

4. The Lens

The first description of cataract due to an amino-acid deficiency was that of Curtis, Hauge and Krayhill (1932), who stated that, in their rats deficient in tryptophan, there occurred a "white opaqueness of the eye and loss of the characteristic colours of the eye." This description alone would hardly have justified the conclusion that they were describing cataract, but their photographs leave no room for doubt. Totter and Day (1942) later confirmed this work, and, subsequently, a deficiency of each of the essential amino acids with the exception of arginine (Hall *et al.*, 1948) was shown to cause lens changes in the rat, although usually not amounting to clinical cataract. In the pig fed a diet primarily characterized by lack of tryptophan, cataract has also been induced (Cartwright *et al.*, 1945).

Recently, in the Human Nutrition Research Unit in London, many of the changes which occur in human protein malnutrition have been produced and studied in pigs fed low protein diets (Platt, 1958). The eyes of fourteen of these pigs were examined and, besides the changes in the para-ocular glands already referred to (p. 38), the only other abnormality, but of the greatest interest, was the development of cataract (McLaren, 1959*b*). This occurred bilaterally in two pigs which survived more than one year on the low protein diet. The appearance of an eye of one of these pigs when 16 months old is shown in Plate III (*a*). On sectioning, the lens showed gross disorganization of the lens fibres, most marked in the subcortical region, with hydropic degeneration of many of the fibres, giving rise to the appearance described in other forms of cataract as "bladder," or "bubble" cells. The nuclei of the equatorial bow were extremely pyknotic, and the large interfibrillar clefts contained much debris (Plate III (*b*)).

The work of Schaeffer and Murray (1950), in which they showed that tryptophan had no specific role in the lens apart from that in protein synthesis, has already been referred to (p. 36). Their conclusion was based upon experiments in which they gave the amino acid, by the method of delayed supplementation, in relatively large quantities, still produced cataract, and found no difference in the tryptophan content of lenses of normal and tryptophan-deficient rats.

Larvae of the tiger salamander (*Amblystoma tigrinum*) developed cortical cataract when fed diets deficient in cystine (Patch, 1934, 1941). It was claimed that the lens changes were of two kinds: the first, produced by diets of prepared casein and powdered milk, or muscle protein treated with dilute alkali and prevented by cystine; and the second, associated with a high haemoglobin ration, not prevented by cystine, but in which the feeding of the three amino acids of

(1922), deficiency of the former was shown to be responsible for the eye changes.

About this time, several theories were put forward to account for the eye lesions. Some held that they were mainly due to infection (Wason, 1921; Yudkin, 1922; and Yudkin and Lambert, 1923), while Mori (1922*a*, 1922*b*) regarded these as being secondary to atrophy of the para-ocular glands and the consequent drying up of their secretions. Findlay (1925) found a *diminution in the* concentration of lysozyme in the glands of vitamin-A-deficient rabbits, and that treatment with normal tears prevented keratomalacia.

However, in 1925, Wolbach and Howe, by their classical study in the rat, showed conclusively that withdrawal of vitamin A was the primary cause of the changes which they demonstrated in, not only cornea, conjunctiva, and para-ocular glands, but also in many other epithelial tissues of the body. In spite of this, *there still persists in the minds of some, probably* because of the interpretation of xerophthalmia as "dry eye," the belief that drying up of the ocular secretions is the essential change. Histologically, the affected epithelia, whether of ectodermal, mesodermal or endodermal origin, undergo keratinization with metaplasia of their cells. Briefly, the changes consist of atrophy, reparative proliferation by basal cells, and growth and differentiation of the new cells into a stratified keratinizing epithelium. Regardless of its origin, this replacement epithelium is comparable in all its layers with epidermis, and returns to normal when vitamin A is administered. Subsequently, the same changes have been demonstrated in numerous other species (Wolbach, 1954); most recently in the pigeon (Mouriquand *et al.*, 1955) which is very resistant to vitamin deficiency, and in the hamster (Salley and Bryson, 1957).

A recent contribution to the study of the pathology of vitamin-A deficiency is the demonstration under the electron microscope, by Sheldon and Zetterqvist (1956), of the derivation of keratohyaline granules from degenerating mitochondria in the corneal epithelium of the vitamin-A-deficient mouse.

Little attention has been paid to Descemet's endothelium and its membrane in vitamin-A deficiency, although it is well known that if they suffer even slight damage, the whole of the cornea is seriously affected. Ramalingaswami, Leach and Sriramachari (1955) found mild degenerative endothelial changes in one eye of one monkey out of three deficient in vitamin A. In this connexion mention may be made of a localized endothelial hyperplasia which the writer encountered in rats deficient in vitamin A and receiving different levels of protein (Plate IV (*a*)). This change could not be related to the level of protein, and did not occur in any of hundreds of control rats which have been examined. When the experiment was repeated on two separate occasions this change did not recur, and no explanation of the aetiology can be offered.

In these same rats, another obscure change occurred in more than 75 per cent of the animals, which also was not again observed when the experiment was repeated on two occasions. It consisted of a subepithelial infiltration of mononuclear cells, taking various forms which, on slit-lamp microscope examination, appeared either as small punctate or finger-like areas, or else as larger confluent granular masses (Plate IV (*b*)).

Mellanby (1931) at first considered the corneal changes of vitamin-A deficiency to be secondary to trigeminal nerve degeneration, and subsequently showed that many other nerves, as a result of pressure from bony overgrowth, were affected. *Wolbach and Bessey (1941), on the other hand,* produced evidence that disproportionate growth of the nervous system, as compared with the surrounding bone, resulted in a gradually increasing pressure on the nerves. Controversy over this intriguing problem is not yet closed, but as it is not strictly pertinent here the interested reader is referred to the monograph of Moore (1957) for a summary of the present position. That vitamin A is capable of bringing about remarkable changes in the morphology of the epithelial cell by acting directly on the epithelium without any mediation of peripheral nerves was demonstrated by the tissue culture experiments of Fell and Mellanby (1953).

The external appearance of the eye in experimental vitamin-A deficiency has been well described by Yudkin (1922). The first sign is increased lachrymation accompanied by photophobia. The eyes then begin to *recede, and the facies becomes sleepy in appearance. Collins (1930) has suggested that this is* due to atrophy and necrosis of the secreting epithelium of the Harderian gland which lies immediately behind the eyeball. The secretion of the glands is more viscid, the lids become oedematous, the rat rubs them with its front paws, and their hair falls out. This gives rise to "spectacle eye" (Plate IV (*c*)) which is also found in some other deficiency states (p. 44). Fatty patches of secretion, which are easily removed from the cornea, accumulate in the fornices, and fluorescein shows that, at this stage, the underlying cornea is intact. Later, porphyrin pigment from the Harderian gland becomes encrusted (Plate IV (*d*)) around the eyes and the lids frequently stick together. By this time the rat has usually ceased to put on weight, frequently has black watery stools, and is a sick animal, often dying from the systemic effects of the deficiency before xerophthalmia has occurred. In the event of the rat surviving

sufficiently long, xerophthalmia supervenes rapidly with drying, infiltration and vascularization, softening of the cornea, and frequent rupture, leading to infection and destruction. The rat has almost no bulbar conjunctiva, but in the rabbit, with an eye more nearly resembling the human in this respect, Hetler (1934) described Bitôt's spot-like lesions, and Mann *et al.* (1946) found there was early outward migration of chromatophores from the limbal pigment ring.

2. The Retina

The earliest changes in the eye take place in the retina, and are readily detectable in man by tests of visual function under conditions of dark adaptation. In experimental animals, also in very young humans, of course, such examinations are much more difficult. As long ago as 1907 it was noticed by Hess that hens, after a stay in the dark, would eat in light less intense than that in which they had stopped eating previous to exposure to darkness. Some time later it was demonstrated (Fridericia and Holm, 1925; Holm, 1925), that vitamin-A-deficient rats suffered from night blindness. Animals were made to jump across a gap to the safety of their cage, and those deficient in vitamin A would behave normally only when either the intensity of the light was increased, or when they were given vitamin A. In 1931, Tansley found that the retina of vitamin-A-deficient rats contained less rhodopsin than that of normal rats of the same strain. The same worker (1933) and later Johnson (1943) described degenerative changes in the rods and nuclear layers of the retina of the rat, and, more recently, similar results have been obtained in the monkey (Ramalingaswami *et al.*, 1955). However, the very careful work of Mann *et al.* (1946) failed to show any such changes in the rabbit retina in vitamin-A deficiency, and they point out that retinal degeneration occurs not uncommonly in inbred rat stocks and may constitute a pitfall in interpretation.

Study of the electroretinogram in vitamin-A deficiency in both rats (Charpentier, 1936) and rabbits (Waters, 1950) has shown a reduction in the amplitude. A rise in the final rod threshold, measured in this way in rabbits, could be detected before any corneal signs were visible. The electroretinogram was restored to normal by the administration of vitamin A. The possible importance of this examination as a means of detecting early vitamin-A deficiency in young children has been mentioned already (p. 31).

An important study of the relationship between night blindness and vitamin-A deficiency has recently been made by Dowling and Wald (1958), using male weanling rats depleted of the vitamin. The exhaustion of liver stores was followed by a precipitous fall in the plasma level to zero. After this, the rhodopsin content of the retina began to fall and was accompanied by a rise in the electroretinogram threshold. Later still, there was a fall in the retinal opsin content and histological changes in the outer segments of the rods, but not before signs of deterioration in the general condition of the animals had appeared.

3. Congenital Malformations

The pioneer work in the field of congenital malformations induced in the offspring by maternal dietary deficiency was that of Hale (1935), in which he fed sows diets low in vitamin A for 150 to 200 days before breeding and during the first days of gestation. Prominent among the various defects which resulted were arrest of development of the eyes and even complete anophthalmos. Warkany has made this field peculiarly his own and, working with the rat, has produced changes in many organs, including the eye, by various deficiency states of which vitamin-A deficiency was the first (Warkany and Schraffenberger, 1946). In outline, these changes consisted of: absence of the anterior chamber, iris, and ciliary body; failure of the vitreous to form, its place being taken by connective tissue which entered the eye through a cleft in the lower half of the retina; and the retina completely disorganized and having the appearance of cerebral tissue. Warkany (1954) found that the incidence of eye lesions was higher when vitamin-A deficiency was maintained throughout pregnancy than when vitamin A was given from the fifteenth day, being reduced from 92 to 68 per cent by this measure. If the vitamin was given from the thirteenth day, only two days earlier, a much greater reduction in the incidence was obtained, to only 15 per cent. Deficiency of vitamin A thus seems to have its most marked effect on the eye at a later stage than folic acid deficiency (p. 46).

Lamming *et al.* (1954) show pictures of "open eye" which occurred in the foetuses of 2 out of 42 rabbits which were fed on a diet containing no detectable carotene.

4. Sex Differences

It was noted earlier (p. 30) that xerophthalmia is more common in the male. The evidence for this is considerable and consistent in the case of man, but is meagre and inconsistent from animal work. Thus, Mayer and Krehl (1948) found that male rats deprived of vitamin A showed signs of deficiency before females, but Booth (1952) was unable to confirm this. So far as mortality was concerned, Coward (1942) found that, from analysis of a large accumulation of data, there was a highly significant greater mortality among

males. Vitamin-A metabolism is clearly different in many respects in the two sexes, and present knowledge on this complex subject has been summarized recently (Moore, 1957).

In an experiment designed to study the effect of protein deficiency on the onset of xerophthalmia, and survival time in the albino rat, it was found (McLaren, 1959a) that female rats took longer to develop xerophthalmia, and survived longer than male rats whatever the level of protein in the diet. A consideration of the growth rate of these animals seemed to indicate that it was an important factor, rapid growth being associated with early onset of xerophthalmia and death.

D. Deficiency of Vitamins of the B Complex

In this section the effects of a deficiency of these vitamins will be considered together as they affect different parts of the eye and its related glands.

1. The Ocular Glands and the Lids

Much of the experimental work on nutritional deficiency has used the rat which, like all animals possessing a third eyelid, has a large gland of Harder situated behind the eye. Derrien and Turchini (1924) showed that the secretion of this gland contains porphyrin, and this is responsible for the bright brick-red fluorescence which the gland exhibits in ultra-violet light. McElroy *et al.* (1941) demonstrated that the pigment responsible for the so-called "blood-caked whiskers" secretion is coproporphyrin. The same appearance has been described in the rat in deficiency of pantothenic acid (Oleson *et al.*, 1939), riboflavin (Bessey and Wolbach, 1939), nicotinic acid (Krehl, 1949) and in water depletion (Figge and Atkinson, 1941).

Denudation of the eyelids of rats deficient in vitamin A was noted by Yudkin (p. 42) in 1922. Sherman and Sandels (1931) gave the name of "spectacle eye" to the same condition which occurred as part of a general dermatitis in vitamin-G-deficient rats. Later György and Eckardt (1940) showed that deficiency of pyridoxine could be responsible. Irinoda and Mikami (1958) have recently described "spectacle eye" in rabbits deficient in pyridoxine and have given an account of the histological appearance of the lids. The writer has seen "spectacle eye" in biotin deficiency induced by avidin, and is of the opinion that it is non-specific, like many other eye signs.

The riboflavin content of the lachrymal and Meibomian glands of the ox is higher than that of any other ocular tissue (Philpot and Pirie, 1943) and it was suggested that the corneal epithelium might obtain some of its riboflavin from the eye secretions.

In riboflavin deficiency in the rat, porphyrin incrustation of the lids occurs (Bessey and Wolbach, 1939), and the Harderian gland shows dense infiltrations of darkly staining lymphocytes, which replace glandular tissue.

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2. The Conjunctiva and the Cornea

As most of the work on the effects of B-vitamin deficiency has been with the rat, attention has been concentrated upon the cornea, for in this species the bulbar conjunctiva is nothing more than an inconspicuous narrow ring of tissue. Sherman and Sandels, in 1931, reported corneal vascularization in vitamin-G deficiency, and the more detailed description by Bessey and Wolbach (1939), in the riboflavin-deficient rat, raised the interesting possibility of similar findings in man, which has already been referred to (p. 32), and was followed by accounts of the condition in other animals. Thus, Street, Cowgill and Zimmerman (1941) described corneal opacities with the appearance of a deep punctate keratitis in dogs, but did not mention corneal vascularization. However, Potter, Axelrod and Elvehjem (1942) found that in their riboflavin-deficient dogs eye signs appeared in 4–9 weeks and consisted of a purulent discharge, followed in a few days by vascularization and, later, by opacities. In the pig, epithelial changes occurred without vascularization (Patek, Post and Victor, 1941), and in both mice (Lippincott and Morris, 1942) and monkeys (Waisman, 1944) corneal changes were absent. The most recent account is that of Halver (1957) of photophobia and corneal vascularization in the chinook salmon. Johnson and Eckardt (1940) suggested that vascularization was hastened by exposure to sunlight, but Lowry and Bessey (1945) showed that this was not so, and that riboflavin in the cornea is probably in a bound form. Except that the oxygen uptake of the cornea in riboflavin deficiency is diminished, nothing further is known about the biochemical lesion (Lee and Hart, 1944).

Apart from riboflavin, deficiency of only two other vitamins of the B complex has been implicated in

corneal lesions. Bowles *et al.* (1949) reported corneal vascularization in rats deficient in either pyridoxine or pantothenic acid. Musini (1954) described blepharitis, neoformation of vessels of the cornea, keratitis and corneal ulcers and abscesses in pyridoxine-deficient rats, and, in addition to blepharo-conjunctivitis, Irinoda and Mikami (1958) noted corneal vascularization in their pyridoxine-deficient rabbits. However, Ramalingaswami and Sinclair (1953) state that they examined their rats suffering from pyridoxine deficiency with the slit-lamp and found no vascularization.

In addition to the lid changes mentioned already (p. 44) Gilbert and Gillman (1944) found that their rats fed a diet of corn pap and fermented milk for two years had, on slit-lamp examination, "irregular blistered epithelium with evidence of scarring." Transparency of the cornea was further reduced by ingrowth of capillaries which formed a network in the corneal substance.

3. The Lens

A diet deficient in certain members of the vitamin-B complex was reported by Salmon, Hays and Guerrant (1928) to cause cataract in rats, occasionally. In 1931, Day Langston and O'Brien found that 94 per cent of their rats on a riboflavin-deficient diet developed cataract in from 60 to 87 days. Day and his colleagues subsequently found that such a diet also produced a similarly high percentage of lens opacities in wild rats (Langston and Day, 1933), mice (Langston, Day and Cosgrove, 1933) and chicks (Day, Langston and Cosgrove, 1934). This work has been repeatedly confirmed, but other investigators have never been able to obtain the very high incidence of cataract reported by Day. It seems unlikely that differences in the strain of rats could be responsible, but composition of the experimental diet might be, for the substitution of egg albumin for casein completely prevented the onset of cataract in both rats and chicks (Day, Langston and Cosgrove, 1934) and supplementation with cystine appeared to decrease the incidence (*see* p. 40). Another factor found to affect the incidence of cataract in riboflavin deficiency is the rate of growth (Day and Darby, 1936), 63 per cent of rats gaining less than 10 grammes weekly becoming cataractous, while the incidence was 0 per cent among those gaining over 60 grammes. Further confusion resulted when Baum, Michaelree and Brown (1942) demonstrated more consistent production of cataract when traces of riboflavin were present than when the diet was riboflavin-free. Relatively minor dietary changes resulted in marked differences in the incidence of lens damage. The many discrepancies in all this work have never been clarified, and they continue to offer an attractive problem for thorough re-investigation.

It is possible that Day (1934) also induced cataract in the monkey by riboflavin deficiency, but the published data are inadequate for independent evaluation. Lippincott and Morris (1942) noted, in passing, that cataract occurred infrequently in their riboflavin-deficient mice. Wintrobe *et al.* (1944*a*) gave a clear description of cataract in riboflavin deficiency in the pig, and a recent account in suckling pigs is that of Miller *et al.* (1954). "Cloudy lens," as well as corneal vascularization, is mentioned by Halver (1957) in the riboflavin-deficient chinook salmon. The only other member of the B complex, a deficiency of which has been associated with lens changes, is choline. Very recent work on the effect of serotonin creatinine sulphate injected subcutaneously in rats has been reported by McDonald, Robbins and Mallory (1958). Because of evidence that serotonin is inactivated by the liver, they induced fatty change in this organ, going on to cirrhosis by feeding a choline-deficient, high calorie, high fat diet. Among other lesions found was cataract.The precise aetiology of the lens damage is not clear, for cataract has not previously been reported in choline deficiency.

It has long been known that inositol is in high concentration in the lens. Van Heyningen (1957) has recently shown that it is in even higher concentration than three other low-molecular weight, water-soluble organic compounds, which are themselves in high concentration and suspected of playing a part in the metabolism of the lens; namely adenosine triphosphate, glutathione and ascorbic acid. The function of inositol in the body as a whole is unknown, but its investigation in the lens may prove to be one of those fruitful contributions to general knowledge.

4. The Retina and the Optic Nerve

The work on the nervous system lesions in experimental thiamine deficiency has been summarized by Pirie and Van Heyningen (1956). They point out the difficulty of interpreting, especially the earlier work, because of such problems as accompanying inanition, species differences, the prolonged nature of the experiments, and the need for pair-fed controls. It seems clear that chronic thiamine deficiency produces degeneration of peripheral nerves in the rat (Prickett, Salmon and Schrader, 1939; Rodger, 1953; North and Sinclair, 1957), but not in the pig (Wintrobe *et al.*, 1944*b*). Degenerative lesions are readily induced in the pigeon (Swank and Prados, 1942) and this species appears to be exceptional in that acute as well as chronic deficiency of thiamine gave nerve damage, namely degeneration of the central terminations of the

males. Vitamin-A metabolism is clearly different in many respects in the two sexes, and present knowledge on this complex subject has been summarized recently (Moore, 1957).

In an experiment designed to study the effect of protein deficiency on the onset of xerophthalmia, and survival time in the albino rat, it was found (McLaren, 1959a) that female rats took longer to develop xerophthalmia, and survived longer than male rats whatever the level of protein in the diet. A consideration of the growth rate of these animals seemed to indicate that it was an important factor, rapid growth being associated with early onset of xerophthalmia and death.

D. Deficiency of Vitamins of the B Complex

In this section the effects of a deficiency of these vitamins will be considered together as they affect different parts of the eye and its related glands.

1. The Ocular Glands and the Lids

Much of the experimental work on nutritional deficiency has used the rat which, like all animals possessing a third eyelid, has a large gland of Harder situated behind the eye. Derrien and Turchini (1924) showed that the secretion of this gland contains porphyrin, and this is responsible for the bright brick-red fluorescence which the gland exhibits in ultra-violet light. McElroy *et al.* (1941) demonstrated that the pigment responsible for the so-called "blood-caked whiskers" secretion is coproporphyrin. The same appearance has been described in the rat in deficiency of pantothenic acid (Oleson *et al.*, 1939), riboflavin (Bessey and Wolbach, 1939), nicotinic acid (Krehl, 1949) and in water depletion (Figge and Atkinson, 1941).

Denudation of the eyelids of rats deficient in vitamin A was noted by Yudkin (p. 42) in 1922. Sherman and Sandels (1931) gave the name of "spectacle eye" to the same condition which occurred as part of a general dermatitis in vitamin-G-deficient rats. Later György and Eckardt (1940) showed that deficiency of pyridoxine could be responsible. Irinoda and Mikami (1958) have recently described "spectacle eye" in rabbits deficient in pyridoxine and have given an account of the histological appearance of the lids. The writer has seen "spectacle eye" in biotin deficiency induced by avidin, and is of the opinion that it is non-specific, like many other eye signs.

The riboflavin content of the lachrymal and Meibomian glands of the ox is higher than that of any other ocular tissue (Philpot and Pirie, 1943) and it was suggested that the corneal epithelium might obtain some of its riboflavin from the eye secretions. In riboflavin deficiency in the rat, porphyrin incrustation of the lids occurs (Bessey and Wolbach, 1939), and the Harderian gland shows dense infiltrations of darkly staining lymphocytes, which replace glandular tissue.

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E. Deficiency of Other Vitamins and Trace Elements

1. Vitamin C

Although the lens, aqueous and vitreous humours, and the epithelium of the cornea all have high concentrations of ascorbic acid, experimental vitamin-C deficiency has not been shown to cause any abnormalities of the eye, apart from the possibility of its becoming involved in the general tendency for haemorrhage to occur, and wound healing to be impaired. Lack of vitamin C would seem to be responsible for interference with the reaction of the collagen of the cornea to injury. Campbell and Ferguson (1950) found that corneal vascularization resulting from thermal burns in scorbutic guinea-pigs was more marked than in control animals. It would seem that the cornea of scorbutic guinea-pigs reacts to cortisone given systemically differently from that of normal controls. Barber and Nothaker (1952) reported that in control animals given cortisone, wound healing was delayed, due to the belated appearance of fibroblasts and prolonged immaturity of the collagen. Similar treatment of scorbutic animals with cortisone failed to change the effects of vitamin-C deficiency, and definite increase in fibroblastic proliferation resulted. According to Boyd (1955) wounds of the cornea take longer to heal in aphakic than in normal eyes, and the suggestion is made that this is possibly due to a lowering of the local concentration of ascorbic acid when the lens is absent.

2. Vitamin D

Here it need only be mentioned that Knapp (1939, 1943), the exponent of "scleral rickets" as the cause of myopia, claimed to have produced prominence of the eyes, wide palpebral fissures, ectasia of the cornea, and deepening of the anterior chamber in dogs deficient in vitamin D and calcium. These results have never been confirmed.

3. Vitamin E

Vitamin E seems to have its main importance, so far as the eye is concerned, in utero. Usually, vitamin-E deficiency in pregnant rats results in foetal absorption, but if supplements of the vitamin are given during mid-pregnancy, some of the embryos damaged during early organogenesis are saved. Callison and Orent-Keiles (1951) reported that the offspring of rats which were deficient in vitamin E had small eyes, and "opaque membranes" behind the pupil, and the eyelids failed to open. Cheng and Thomas (1955) also described ocular abnormalities in the young of rats deficient in vitamin E. Ferguson (1956) has described cataract and keratoconus-like changes in the cornea of the embryos from turkey hens which were deficient in vitamin E. It was claimed by Demole and Knapp (1941) that they had produced kerato-conjunctivitis regularly, and other eye lesions such as corneal vascularization and cataract exceptionally, in rats fed a diet deficient in vitamin E. They did not give details of their diets and methods, and these changes have not been reported by others.

Before the aetiology of retrolental fibroplasia was known, vitamin-E deficiency was one of the more popular suggestions, and although the major role of of the abuse of oxygen therapy in prematurity is now clear, it is still possible that the known anti-oxidant effect of vitamin E may be involved in some way. That the general state of nutrition may influence the growth of retinal vessels induced by exposure to oxygen has been shown by Hellström (1956) using the mouse.

4. Essential Fatty Acids

In an advanced stage of essential fatty-acid deficiency in rats, an erythema and scaliness of the eyelid with accompanying conjunctivitis has been observed (Alfin-Slater, personal communication). Presumably this is a further instance of "spectacle eye" in the malnourished rat.

5. Calcium

Von Bahr (1936) showed that the calcium-phosphorus ratio was all-important in the production of cataract. Rats fed a rachitic diet with a calcium-phosphorus ratio of 4:1, the proportion generally employed in the dietary production of rickets, showed no lens changes. Upon replacing this diet with a normal stock diet (Ca:P=1:1) cataract resulted. Of 109 rachitic rats, 49 developed cataract, and of these, all but one had received the stock diet. The change to this diet also caused a lowering of the blood calcium with tetany. Goldmann (1929), in experimental animals, produced tetanic attacks, alternating with periods free from tetany, and observed under the slit-lamp that opaque zones of fibres coinciding with the tetanic attacks alternated with clear fibres produced during the tetany-free periods. He concluded that the cataractous changes resulted from acute poisoning of lens fibres and not from a chronic state of hypocalcaemia.

6. Sodium

A diet containing only 0·002 per cent of sodium was devised by Orent-Keiles, Robinson and McCollum (1937). After 6 or 8 weeks on such a diet, rats began to show eye changes which consisted of "sanguinolent secretion" (presumably porphyrin in nature), "corneal ulceration, perforation, hypopyon and keratinization,

optic nerve fibres and secondary optic centres. Rodger (1953) found that about half of the rats suffering from chronic thiamine deficiency showed changes in the optic nerves, the lateral geniculate body, and the dorsal nucleus, but the retina was unaffected. He went on to show (Rodger, 1954) that in a chronic deficiency of both thiamine and riboflavin, the rats lived longer than those deficient in thiamine alone, and all showed degenerative changes in the optic tract. North and Sinclair (1957), in a study of the effects on the nervous system of the rat of acute episodes of thiamine deficiency superimposed upon chronic pantothenic acid deficiency, found no changes which were not also seen in inanition-control rats. The optic pathway was unfortunately not examined. They attributed the finding of degeneration in much of the earlier work, in which the Marchi method or the appearance of myelin under polarized light were relied upon entirely, to changes due to inanition, and advocated the use of osmium tetroxide and Sudan black.

In view of the disputed aetiology of nutritional amblyopia in man, one of the most profitable studies in experimental animals in this field would seem to be to observe the histological changes in the optic pathway in isolated and combined deficiency of some of the other B vitamins, such as folic acid and vitamin B_{12}.

Riboflavin has been found in the retina of many species. Pirie (1943) found only small quantities in the mammalian retina, and this was nearly all bound as riboflavine–adenine–dinucleotide, which is light-stable, and clear evidence that riboflavin acts as a photosensitizer in the mammalian retina is lacking. It has been shown by Wald (1953) that nicotinic acid is involved in the rhodopsin system of the retina as the functional component of diphosphopyridine nucleotide (DPN) aiding the conversion of retinene to vitamin A by hydrogen transfer.

The effects of choline deficiency on the eye are on the hyaloid arterial system, but may be conveniently mentioned here. It was noted by Griffith and Wade (1939) in describing choline deficiency in rats, that intra-ocular haemorrhages occurred in those animals in which the kidneys were most severely affected. Bellows and Chinn (1943) found that the most frequent lesion was a column of blood in Cloquet's canal, which is formed round the hyaloid artery, the artery supplying the lens in foetal life, but which disappears shortly after birth in the rat. In puppies, no eye changes were found. This species difference was explained by Burns and Hartroft (1949), who showed that the haemorrhages arose by diapedesis from the hyaloid arterial system, which had to be patent at the time of the deficiency. In puppies and mature rats, the system had closed and intra-ocular haemorrhages could not occur, moreover damage to the kidney is less severe than in the weanling rat.

5. Congenital Malformations

In recent years, a deficiency of many of the vitamins of the B complex has been implicated in the production of congenital defects, and in most instances the eye has been one of the organs affected. Nearly all this work has been done with the rat.

In the early work of Warkany (1945) with riboflavin deficiency, cerebral or eye defects were observed only rarely. Recently, using the riboflavin antagonist galactoflavin, Nelson *et al.* (1956) have induced multiple congenital abnormalities, including "open" eyes and microphthalmia with a higher incidence. Depending on the length of the deficiency period and the dose of galactoflavin, cerebral and eye defects together were found in 7 and 9 per cent of two of their groups. Grainger, O'Dell and Hogan (1954) reported as high an incidence as 15 per cent, but this was markedly reduced by adding vitamin B_{12} to their riboflavin-deficient diet. It would seem, then, that most of the responsibility for causing the "small or missing eyes" in their work must be laid at the door of vitamin B_{12} deficiency.

Using a folic acid antimetabolite, Nelson *et al.* (1955) had earlier produced by a transient maternal folic acid deficiency in rats the following, among many other malformations in the offspring: cataract, coloboma and eversion of the retina, and microphthalmia and anophthalmia. A 48-hour period of maternal deficiency, during days 7 to 9, occurring just after the implantation of the embryo, was sufficient to result in a high incidence of these anomalies. Thus, folic-acid deficiency seems to have its maximum effect upon the eye several days earlier than that of vitamin-A deficiency (p. 43).

Giroud and Boisselot (1947) first showed that deficiency of pantothenic acid during gestation in the rat may result in malformations which include microphthalmos and anophthalmos.

One report appears to implicate nicotinic acid, and especially in its relationship to tryptophan metabolism, in the production of congenital cataract in the rat. Pike (1951) found that a maternal diet containing either: (i) 0·2 per cent tryptophan and no nicotinic acid, or (ii) 10 mg per cent nicotinic acid and only 0·025 per cent tryptophan caused cataracts in some of the offspring, but that 0·2 per cent tryptophan and 10 mg per cent nicotinic acid prevented their occurrence.

Finally, up until the time of writing, congenital malformations involving the eye have not been reported in the young of animals deficient in any of the other members of the vitamin-B complex.

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but with the normal amount of tears." Later, the histological changes were described by Follis, Orent-Keiles and McCollum (1942). These consisted of dilatation of the ducts of the tarsal and Meibomian glands, but with no changes in the lachrymal glands, and in the Harderian gland, only just before the end of the experiment. In the cornea, there was vascularization, infiltration of the stroma with leucocytes, and keratinization of the epithelium.

No eye changes were observed over a period of eight weeks in dogs fed a diet deficient in sodium (Turpeinen, 1938).

7. Phosphorus

In an early paper it was claimed by Yudkin (1924) that the absence of both phosphorus and vitamin A from a diet, led to a more rapid onset of keratomalacia in rats than when vitamin A alone was withheld.

8. Zinc

Follis, Day and McCollum (1941) described corneal vascularization, without keratinization, in two out of seven rats which were fed a diet supplying only 2 to 4 μg per rat per day of this element.

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3

Dietary Factors and Adrenocortical Hormone Secretion

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zona glomerulosa appears essentially normal, the zona fasciculata is thickened and the normal cellular pattern disorganized. Histochemical and biochemical studies of the adrenal cortex revealed a loss of cholesterol and sudanophilic material, and a marked reduction in the number of birefringent particles present (Stepto *et al.*, 1951). These alterations in lipid distribution have been interpreted, by some, as evidence of altered steroid hormone secretion.

4. Vitamin-C Deficiency and Adrenocortical Hormone Secretion

The occurrence of adrenal hypertrophy in scurvy, and the decrease in ascorbic acid associated with cortical hormone secretion, suggested that vitamin C might be concerned with production of adrenal steroid hormones. These findings prompted many studies which were designed to elucidate the relationship of ascorbic acid to function of the adrenal cortex. Giroud and Ratsimamanga (1940, 1941) concluded that decreased adrenocortical hormone secretion occurred in scorbutic animals, but their view was not widely accepted since non-specific methods of determining cortical hormones were used. Shortly after, it was demonstrated that a decrease in number of circulating eosinophils is a sensitive index of cortical hormone secretion. Eisenstein and Shank (1951) used this method to study adrenal function in scorbutic guinea-pigs. These workers found that, even though the ascorbic acid content of adrenal tissue was reduced to approximately 1–2 per cent of the control level, the gland continued to secrete steroid hormones in response to ACTH. As a result of this investigation, it was suggested that vitamin-C deficiency is a non-specific form of stress, and that the vitamin is not directly involved in synthesis or release of adrenocortical hormones. Other experimental evidence which supported this view was provided by Nadel and Schneider (1952), who showed a progressive rise in excretion of formaldehydogenic steroids by guinea-pigs during development of scurvy. Further substantiation came from studies of adrenal steroid hormone levels in plasma of scorbutic animals. Done *et al.* (1953) found that in late scurvy, blood 17-hydroxycorticosteroid concentrations were very much higher than those of control animals. Recent experimental work has shown an increase in plasma corticoid levels during early stages of ascorbic-acid deficiency. As a matter of fact, the rise occurred well in advance of cessation of weight gain (Jones, Peric-Golia and Eik-Nes, 1958). The increase in plasma and urine corticosteroid levels in scorbutic animals is certainly a result of increased hormone secretion, since Jones *et al.* (1958) showed that inactivation of adrenal steroids is not impaired in vitamin-C deficiency.

The results of these studies strongly indicate that vitamin C is not essential for synthesis of adrenal steroid hormones. Further support for this impression is obtained from the work of Guillemin *et al.* (1958), who showed that administration of very small doses of ACTH, to rats, results in a prompt increase in corticosterone secretion even though there is no change in adrenal ascorbic acid concentration. If vitamin C is not essential for hormone synthesis, what is the significance of the high concentration of this vitamin in the adrenal cortex? Recent studies (Bacchus, 1957*a*, 1957*b*) suggest that in ascorbic-acid deficiency there is decreased activity of certain enzyme systems concerned with cortical hormone biosynthesis. On the other hand, investigations of hormone levels in blood and urine of scorbutic animals have shown that cortisol secretion is unimpaired. If vitamin-C deprivation altered enzymatic reactions which participate in steroid synthesis, the secretion of cortisol would undoubtedly be affected. It may be that ascorbic acid is involved in adrenocortical hormone synthesis by participating in certain oxidation-reduction reactions, but that in the absence of this vitamin some other substance carries on this function.

III. PANTOTHENIC ACID

1. Distribution and Metabolic Functions of Pantothenic Acid

Although the distribution of pantothenic acid in nature is ubiquitous, this water-soluble vitamin does not exist in free form in the animal body. Instead, most of it occurs in combination with adenylic acid, phosphate, and thioethanolamine as co-enzyme A. Co-enzyme A (Co A) is concerned with a variety of metabolic processes, since it acts as a carrier of acetyl groups, and to catalyse the transfer of these two carbon molecules to certain acceptors. It has also been shown that Co A serves to activate and transfer acyl groups other than acetate. This co-enzyme is necessary for synthesis of acetylcholine, citric acid, and many other substances. It is concerned with inactivation of drugs and chemicals such as sulphanilamide and para-aminobenzoic acid. Co A is essential for the metabolic degradation of fatty acids as well as the biosynthesis of these compounds (Novelli, 1953; Ralli and Dumm, 1953).

Since pantothenate is present in animal tissues primarily in the form of Co A, studies of tissue con-

measuring the rate of steroid hormone production. A number of studies concerning the relationship of nutritional factors to the adrenal cortex have been conducted utilizing these newer methods of hormone analysis.

This article is not intended to be a review of all experimental data relating nutritional factors to adrenocortical function. Instead, the discussion will deal primarily with those areas in which the author has conducted experimental studies. In each instance in which the role of a specific nutrient in adrenal cortical function is considered, an effort has been made to include results of those investigations which contribute significantly to our current understanding of the problem.

II. ASCORBIC ACID

1. Presence of Ascorbic Acid in the Adrenal Cortex

Ascorbic acid was first isolated from animal tissues by Szent-Gyorgi (1928) who found the vitamin in significant quantities in the adrenal gland. It was subsequently shown that ascorbic acid concentration in this gland is greater than that of any other tissue (Morgan, 1951). Vitamin C is present in both the adrenal medulla and cortex, but it has been established that the cortex contains the larger amount (Bourne, 1933). Cytochemical studies of the distribution of ascorbic acid in cortical tissue have revealed that the vitamin is present in cytoplasm of virtually all parenchymal cells. In the zona glomerulosa, however, the concentration of vitamin C does not appear to be as great as that of the zona fasciculata or zona reticularis (Deane and Morse, 1948).

2. Changes in Adrenal Ascorbic Acid Associated with Steroid Hormone Secretion

Many observations have shown that there is often a reduction in adrenal ascorbic acid concentration associated with increased adrenocortical activity. Sayers and Sayers (1948) demonstrated that administration of a single dose of ACTH to rats results in a prompt decline of adrenal ascorbic acid. Within 12 hours, however, the original concentration of this vitamin in cortical tissue is regained. Similar changes in ascorbic acid levels occur in guinea-pigs and other animals following ACTH administration. It has been noted, however, that reaccumulation of vitamin C in the adrenal occurs more slowly in guinea-pigs than in animals which are capable of endogenous synthesis of this vitamin. Other studies (Morgan, 1951) demonstrated that a great variety of stresses such as diphtheria toxin administration, cold exposure, ether inhalation, anoxia and haemorrhage will produce a significant depletion of adrenal ascorbic acid just as does ACTH. In certain instances it has been possible to show that a relationship exists between intensity of the stressful procedure and the absolute decrease in concentration of ascorbic acid (Sayers and Sayers, 1948). The decline in adrenal ascorbic acid following ACTH administration has been used as a method of assay for this trophic hormone (Sayers, Sayers and Woodbury, 1948).

Until recently, the fate of ascorbic acid which disappears from the adrenal subsequent to stress or ACTH was not known. Slusher and Roberts (1957) found that the ascorbic acid content of adrenal venous blood increases after ACTH. These authors state that the vitamin C which is lost from adrenal tissue can be quantitatively recovered from the effluent blood. Their findings also showed that, while ascorbic acid left the adrenal very quickly after ACTH stimulation, the maximal rate of hormone release was not attained for 15 to 30 minutes. Other investigators (Munson and Toepel, 1958) found, during short experiments, that the increase in adrenal vein ascorbic acid was greater than the loss of vitamin C from adrenal tissue. It was suggested, therefore, that ACTH may not only trigger a discharge of ascorbic acid from the adrenal but may also increase the rate of biosynthesis of the vitamin. This seems unlikely, however, in view of the observations of Grollman and Lehninger (1957), who showed that in most animal species ascorbic acid is not synthetized in the adrenal.

3. Morphological Alterations of the Adrenal in Scurvy

Adrenal hypertrophy was recognized as a manifestation of scurvy before vitamin C was discovered. The work of Bessessen (1923) and others demonstrated that the adrenals of ascorbic-acid-deficient guinea-pigs were considerably enlarged. More recently, an experiment was conducted by Eisenstein and Shank (1951) in which guinea-pigs were fed diets which provided varying levels of ascorbic acid. Their observations showed that there was an inverse relationship between vitamin C intake and adrenal weight. It was postulated that ascorbic-acid deficiency is a form of stress and, as such, causes adrenal hypertrophy similar to that which results from other forms of chronic stress.

The histological appearance of the adrenals of scorbutic guinea-pigs is likewise altered. Although the

quantitatively determined by chemical analysis. The results demonstrated that secretion of hormones by the adrenals of pantothenic-acid-deficient rats was significantly less than that of controls. This observation was soon corroborated by Longwell, Reif and Hansbury (1958), who studied adrenocortical function in pantothenate-deficient and control rats by measuring hormone content of adrenal vein blood. Adrenal venous blood was obtained by cannulation, and the concentration of individual steroids was estimated. It was found that production of corticosterone, the predominant corticosteroid secreted by the rat adrenal, was significantly reduced by the vitamin deficiency. Further confirmation of the important role of pantothenic acid in synthesis of adrenal cortical hormones was obtained from the recent work of Eisenstein and Wenneker (1958). These investigators induced the vitamin deficiency in young guinea-pigs by feeding a deficient diet to which an antagonist of pantothenic acid, omega-methyl pantothenate, was added. The urinary excretion of 17-hydroxycorticosteroid hormones by animals on the deficient diet was determined and compared with that of controls. Adrenal hormone excretion was followed from the onset of the study until vitamin-depleted guinea-pigs were severely ill. Several times during the experiment, ACTH was administered to animals of both groups, to determine responsiveness of the adrenal cortex as the deficiency developed. It was found that ACTH administration to control guinea-pigs resulted in a progressive increase in corticoid excretion, but, in deficient animals, steroid hormone excretion remained at a constant and significantly lower level.

The results of the experiments cited have clearly established that elaboration of adrenal steroid hormones is dependent on adequate body stores of pantothenic acid. The mechanism by which this vitamin acts to facilitate cortical hormone production has not been elucidated, but its function must be identical with that of Co A since this is the major form in which pantothenic acid exists in the body. Co A serves as a carrier and activator of acyl groups and is essential for production of various complex substances, including cholesterol and steroid hormones, from simple precursors such as acetate. Pantothenic acid is an integral part of Co A, and a deficiency of this vitamin leads to depletion of body stores of the co-enzyme. Since the concentration of Co A in adrenal tissue is reduced, it is not surprising to find decreased synthesis of steroid hormones in animals deficient in pantothenic acid.

IV. VITAMIN B_6

1. Structure and Function of Vitamin B_6

It was originally thought that vitamin B_6 and pyridoxine were synonymous terms; however, further investigation has shown that other forms of the vitamin, namely pyridoxal and pyridoxamine, also occur (Snell, 1958). As a matter of fact, these latter forms are more abundant and more widely distributed in nature than is pyridoxine. The three substances are considered to be of equal importance and the group is designated as vitamin B_6.

Vitamin B_6 has been found to act as a co-factor in certain enzymatic reactions. The most active form of the vitamin is pyridoxal phosphate, although a phosphate ester of pyridoxamine has also been shown to possess vitamin activity. Pyridoxal phosphate serves as a co-enzyme for several different reactions involving alpha-amino acids as substrates. It was first shown to be concerned with decarboxylation of tyrosine, and, subsequently, other amino acid decarboxylases were demonstrated to contain this vitamin (White *et al.*, 1954). In studies of growth factors for micro-organisms, pyridoxal phosphate was demonstrated to be the co-enzyme [illegible]
reactions. [illegible]
serves a [illegible]

which occur in animal tissues (Schlenk and Snell, 1945). Pyridoxal phosphate is also known to be the co-factor for other enzymes, including racemases and desulphhydrases (Snell, 1958).

2. Alterations of the Adrenal Cortex in Vitamin-B_6 Deficiency

In studies of the effects of various vitamin deficiencies on the rat adrenal, Deane and Shaw (1947) found that pyridoxine depletion induced certain alterations in this gland. As early as 3 weeks after animals were placed on the deficient diet, there was an increase in the adrenal weight–body weight ratio. Cytochemical studies at this time showed a normal zona glomerulosa, but an increased amount of lipid material in the fascicular and reticular zones. Furthermore, these zones were filled with small birefringent particles. After more prolonged deficiency, however, the histologic appearance of the adrenal cortex was not significantly different from that of controls, although the gland remained hypertrophic. On the basis of these observations, the authors concluded that pyridoxine deficiency did not result in altered adrenocortical hormone secretion. This problem was re-investigated by Stebbins (1951*a*), who induced the

centration of the co-enzyme provide information as to the distribution of pantothenic acid. Kaplan and Lipman (1948) investigated the Co A content of various organs and have shown that, next to the liver, the adrenal gland has the highest concentration of this substance. Their studies demonstrated that most of the adrenal Co A is present in the cortex. Malström and Glick (1954) studied the distribution of Co A in the adrenal by measuring the concentration of this co-enzyme in microtome sections taken from various parts of the gland. The highest levels were present in the outer portion of the zona fasciculata and the fascicular-reticular zone, with low concentrations being found in the mid-fasciculata and zona reticularis. Greenberg and Glick (1958) found that ACTH administration produced significantly increased concentrations of Co A in the glomerular–fascicular and fascicular–reticular zones, but smaller increases occurred in the zona reticularis and medulla.

2. Alterations in the Adrenal Gland Associated with Pantothenic Acid Deficiency

Attention was first drawn to the relationship of pantothenic acid to the adrenal cortex by Morgan and Sims (1939), who found adrenal haemorrhages in rats that had been fed a diet deficient in "filtrate factor." Shortly thereafter, Daft and Sebrell (1940) found adrenal haemorrhages, hypertrophy and necrosis in pantothenic-acid-deficient animals and were able to correct these abnormalities by administering pantothenate. Their findings were confirmed and extended by Deane and McKibbin (1946), who conducted histochemical studies of changes in the adrenal cortex during the progress of pantothenate deficiency. These investigators found that lipid substances disappeared from the zona reticularis and inner part of the zona fasciculata within a few weeks. Other substances, thought to be ketosteroid in nature, disappeared even more quickly, and the fasciculata and reticularis became completely exhausted of this material although moderate amounts remained in the zona glomerulosa. As the deficiency progressed, areas of necrosis and haemorrhage appeared throughout the gland. The work of other investigators (Krehl, Winters and Schultz, 1952) demonstrated that adrenal lesions resulting from pantothenic acid depletion are intensified when cortical hormone secretion is increased. ACTH administration, for example, results in extensive haemorrhagic necrosis of the gland.

3. Function of the Adrenal Cortex in Pantothenic-Acid Deficiency

The morphological and histochemical alterations of the adrenal in pantothenic-acid deficiency were interpreted by many investigators as evidence of decreased cortical hormone secretion. In particular, the reduction of lipid content of cortical tissue was considered to be significant. Because of these findings, several groups of investigators conducted studies which were designed to assess functional activity of the adrenal cortex of pantothenate-deficient animals. Winters, Schultz and Krehl (1952*a*) followed changes in eosinophil and lymphocyte counts after administration of ACTH and epinephrine to deficient and control animals. It was found that eosinopenia and lymphopenia, which normally occur after injection of these agents, were abolished in pantothenic-acid depleted rats. However, when cortisone was given, there were decreases in the number of eosinophils and lymphocytes in deficient animals, similar to those which occurred in control rats.

These investigators (Winters *et al.*, 1952*b*) also studied carbohydrate metabolism in pantothenate-depleted and control rats. In deficient animals, fasting blood sugar levels were lowered and, in addition, there was a marked increase in insulin sensitivity. Deficient animals also demonstrated impaired ability to synthetize liver glycogen during a fast, or following ACTH. It was possible to correct these defects in carbohydrate metabolism by treatment with cortisone. The experiments of Hurley and Morgan (1952) and Hurley and MacKenzie (1954) supported the observations of Winter *et al.*, since they also showed that stress-induced changes in blood sugar and liver glycogen were abolished in pantothenate-deficient rats. Further substantiation of the idea that adrenal steroid secretion is reduced in pantothenic-acid deficiency was provided by investigations of adrenal cholesterol concentration. These studies showed that, in deficient rats, cholesterol levels were reduced (Winters *et al.*, 1952*c*) and, further, that there was delayed reaccumulation of this sterol in adrenal tissue following stress (Dumm *et al.*, 1953). The observations concerning cholesterol appeared to be of particular importance, since it is known that this substance is a key intermediate in steroid hormone synthesis.

Although these experiments indicated that there is diminished adrenal steroid secretion in pantothenate-deficient animals, the evidence did not appear conclusive, since no direct measurement of cortical hormone production had been carried out. For this reason, Eisenstein (1957) studied the ability of the isolated adrenal cortex of pantothenic-acid-deficient rats to secrete steroid hormones under the stimulus of ACTH. In this experiment, adrenals were removed from pantothenate-depleted and control rats, and incubated in a medium containing ACTH as well as various nutrients. After incubation, steroids secreted into the medium were recovered by extraction, and

reduced, there is increased secretion of salt-retaining hormone by the zona glomerulosa. The investigation also showed that when the sodium: potassium ratio was elevated by administering desoxycorticosterone acetate or by restricting dietary intake of potassium, the zona glomerulosa became narrower and the cells smaller than normal, changes which suggested inactivity. The adrenal effects of altered body electrolyte composition were shown to be independent of the pituitary, since they were readily apparent in hypophysectomized animals.

Peschel and Race (1954) confirmed the findings of Deane *et al.* (1948), in the rat, and contributed significant information concerning the response of the human adrenal to salt restriction. These investigators examined the adrenal glands of hypertensive patients who died while on a salt-free rice diet. They found that, in approximately one-fourth of these patients, there was significant widening of the zona glomerulosa and a decrease in lipid content of the adrenal. Special histochemical techniques showed this lipid material to be ketosteroid in type. Peschel and Race (1954) interpreted their findings as evidence of increased hormone production, and suggested that a decreased sodium: potassium ratio stimulated secretion of electrolyte-regulating corticoids by the zona glomerulosa.

This problem was reinvestigated by Hartroft and Eisenstein (1957) in studies designed to correlate histological appearance of the adrenal with steroid secretion in sodium restricted rats. In these experiments, marked enlargement of the zona glomerulosa was found in association with altered lipid storage. Not only was there an increase in total lipid present, but, also, the lipid was stored in the form of large globules instead of the usual small droplets. Since determination of steroid secretion revealed increased release of aldosterone (Eisenstein and Hartroft, 1957) it seemed likely that the observed histologic alterations represented increased cellular activity. In addition to changes in the zona glomerulosa, severe atrophy of the zona fasciculata was observed in sodium-deficient rats. It was suggested that atrophy of the fasciculata was secondary and probably due to compression from the rapid and marked enlargement of the zona glomerulosa. The authors believe that the failure of other investigators to observe atrophy of the fasciculata is due to the fact that, in previous experiments, sodium restriction was not of sufficient severity.

2. Alterations in Electrolyte Intake and Adrenal Steroid Secretion

The morphological studies of the adrenal described in the preceding section led many investigators to conclude that sodium deprivation stimulates the adrenal to secrete a hormone which has its primary effects on electrolyte balance. Since the recent discovery of the electrolyte-regulating hormone, aldosterone, several groups of investigators have demonstrated that secretion of this hormone is increased whenever sodium intake is lowered. Luetscher and Johnson (1954) found approximately a five-fold increase in urinary aldosterone excretion by normal men after sodium restriction. Studies by Rosnagel and Farrell (1956) showed that severe limitation of sodium intake in the dog leads to an approximate two-fold increase in adrenal secretion of aldosterone. The experiments of Eisenstein and Hartroft (1957) demonstrated an increased secretion of aldosterone by the isolated adrenals of sodium-depleted rats. In addition, secretion of corticosterone, the principal adrenal hormone in the rat, and other steroids was reduced. In these studies a progressive increase in aldosterone secretion was found to be correlated with widening of the zona glomerulosa. Further, as the zona fasciculata became atrophic there was concomitant reduction in corticosterone synthesis and release.

The occurrence of increased secretion of aldosterone by the adrenals of sodium-deprived rats was not surprising, but the finding of reduced production of corticosterone was unexpected. Atrophy of the zona fasciculata and diminished corticosterone secretion in sodium deficiency have not been recognized by other investigators, and the significance of these observations is not clear. It may be that this response to sodium restriction is specific for the rat, and does not occur in other animal species. That this may be true is suggested by the studies of Rosnagel and Farrell (1956), who found no change in hydrocortisone secretion in sodium-deficient dogs, despite a doubling of aldosterone output. Furthermore, studies in humans have shown that sodium deficiency does not result in altered 17-hydroxycorticoid excretion. It should be pointed out, however, that sodium depletion was of great severity in the experiments of Eisenstein and Hartroft, and this may account for the unusual response of the adrenal.

There is also a great deal of evidence which demonstrates that body potassium concentration exerts considerable influence on aldosterone secretion. It has been shown that when potassium content of the diet is lowered, aldosterone production is reduced. On the other hand, aldosterone secretion is increased when large loads of potassium are administered (Bartter, 1956). Laragh and Stoerk (1957) have shown that dietary sodium restriction in dogs does not result in increased urinary aldosterone unless potassium supplements are provided. They regarded the concentration of serum potassium to be more important as a regulator of aldosterone secretion than is the sodium level. Even though it is apparent that potassium concentra-

vitamin deficiency in rats, both by feeding a pyridoxine deficient diet, and by feeding a deficient diet to which a vitamin antagonist, desoxypyridoxine, was added. Within 3 weeks there was hypertrophy of the adrenal, and a marked loss of lipid material and cholesterol from the zona fasciculata and zona reticularis in those animals fed desoxypyridoxine. These changes occurred more slowly in rats given the deficient diet without the antagonist, but were readily recognized after 5 weeks. Stebbins (*1951a*) stated that the discrepancy between his results and those of Deane and Shaw (1947) was probably due to a difference in severity of the nutritional deficiency. This investigator further suggested that B_6 depletion results in reduced secretion of hormones by the adrenal cortex. Recently, in a study of pyridoxine deficiency in the rat, Combridge (1956) found adrenal hypertrophy, cellular hyperplasia in the zona fasciculata, and increased deposits of sudanophilic material in cortical tissue. On the basis of these observations, it was suggested that adrenocortical hormone secretion was increased in vitamin-B_6 deficiency.

The preceding discussion has indicated that there is no unanimity of opinion in regard to histochemical changes which are found in the adrenals of vitamin-B_6-deficient animals. It is not a surprise, then, to find that opinions as to function of the gland based on these observations are contradictory.

3. Function of the Adrenal Cortex in Vitamin-B_6 Deficiency

A number of investigators have studied adrenocortical function in B_6-depleted animals because of the morphological alterations which were observed in this deficiency, also because the importance of pantothenic acid in adrenal function had been recognized. Stebbins (1951*b*) found delayed diuresis after water ingestion, and reduced resistance to water intoxication in B_6-deficient rats. These abnormalities were corrected either by administering pyridoxine or adrenal cortical hormones. These observations were interpreted as indicating adrenal dysfunction as a result of the vitamin deficiency. This idea received some support from the work of Ershoff (1951), who noted decreased resistance to cold in pyridoxine-depleted rats, although it was suggested that diminished ACTH production might also be responsible for the increased mortality. Other workers, however, obtained results which have led to divergent views. Butler and Morgan (1953) studied adrenal steroid secretion in pyridoxine deficiency by following eosinophil levels in peripheral blood after ACTH administration. These authors found a similar response in deficient and control rats, and concluded that adrenocortical function was normal despite B_6 depletion. Similar findings were obtained by Ershoff and Parrott (1953).

Because the role of vitamin B_6 in adrenocortical function had not been clarified, Eisenstein (1959) conducted a series of experiments in which steroid secretion by isolated adrenals of pyridoxine-deficient rats was compared with that of control animals. B_6 depletion was induced in some of these studies by feeding a pyridoxine-deficient diet, but in others, desoxypyridoxine, a vitamin antagonist, was added to the food to accelerate the appearance of manifestations of the deficiency. Marked adrenal enlargement occurred in deficient animals. This hypertrophy was attributed to the vitamin deficiency and was not thought to be due, simply, to partial starvation, since adrenal enlargement was greater in pyridoxine-depleted rats than in pair-fed controls. In contrast to previous studies, adrenocortical function was assessed in this investigation by direct *in vitro* determination of steroid secretion. The results of all experiments demonstrated that hormone production by the adrenals of B_6-deficient rats was equal to that of pair-fed and *ad lib* fed controls, and seemed to justify the conclusion that function of the adrenal cortex is not altered in vitamin-B_6 deficiency.

At this point it should be mentioned that the occurrence of adrenal hypertrophy is not a reliable index of cortical hormone secretion. This is apparent from the foregoing discussion, since enlargement of the adrenal was described in pantothenic-acid depletion, pyridoxine deficiency and scurvy, yet adrenocortical hormone production is depressed in pantothenate-deprived animals, unchanged in B_6 deficiency, and increased in scurvy.

V. SODIUM AND POTASSIUM

1. Morphological Alterations in the Adrenal Cortex Associated with Changes in Body Sodium and Potassium

The studies of Deane, Shaw and Greep (1948) showed that sodium restriction induced marked alterations in the rat adrenal cortex. Progressive widening of the zona glomerulosa, and gradual depletion of lipids from this zone, were observed in intact and hypophysectomized rats during sodium deprivation, but no changes were detected in the zona fasciculata. Similar changes in the adrenal could be produced by an increased potassium intake. On the basis of these observations, it was concluded that when the ratio of body sodium to potassium is

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tion has marked effects on production of the electrolyte-regulating hormone, it has not been established that the body potassium content is the primary factor controlling aldosterone secretion.

The foregoing discussion emphasizes that sodium and potassium levels in the body have profound influence on adrenocortical hormone production, but the mechanisms by which changes in electrolyte concentration alter steroid secretion have not been elucidated. The sodium and potassium content of the blood perfusing the adrenal may determine how much aldosterone is secreted. On the other hand, there may be a centre in the nervous system, or some other part of the body, which responds to electrolyte composition of body fluids or to extracellular fluid volume and, in turn, controls adrenal release of the electrolyte-regulating hormone. Regardless of mechanisms of control, the adrenal cortex responds quickly to changes in intake of sodium and potassium.

VI. SUMMARY

In this article the relationship of several vitamins and minerals to function of the adrenal cortex has been discussed. Although ascorbic acid is present in cortical tissue in high concentration, and the amount decreases when the gland is stimulated, this vitamin does not appear to be importantly related to adrenocortical hormone secretion. The same is also true in regard to vitamin B_6, since hormone production in response to ACTH remains intact in pyridoxine-depleted animals. Pantothenic acid, however, has been shown to be essential for synthesis of the adrenal steroids. The function of this vitamin is probably dependent on the fact that it is an integral part of co-enzyme A. The body content of sodium and potassium also importantly influence adrenal cortical hormone secretion, since changes in concentration of these minerals, in some manner determine the quantity of aldosterone that is produced.

While there is considerable information available concerning the effect of certain nutrients on adrenal function, much investigation remains to be done. There have been few experiments conducted to determine the effect of protein restriction, fatty acid deprivation, and caloric underfeeding on secretion of adrenal steroid hormones. Furthermore, the effect of other vitamin deficiencies on adrenocortical function has not been adequately investigated. Studies of the relationship of dietary substances to function of the endocrine glands is a fertile area for future scientific endeavour.

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4

Microbiology of Digestion

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Contents

I. INTRODUCTION

All animals probably harbour bacteria, and frequently also protozoa, in their digestive tracts, and these micro-organisms are in some cases of benefit to the nutritional processes of the host animal. The benefit to be derived by the host, in general, depends on the structure of the digestive tract and the state of the food eaten. In most higher animals the caecum and colon are the main sites of bacterial action, and in some herbivores such as the horse, the caecum is enlarged to permit of extensive microbial action on the digesta. However, in all these cases microbial action follows the gastric digestion of the food though, as we shall see later, in the rabbit the situation is not so clear-cut. Ruminants, and to some extent other mammals such as the wallaby, differ from most others in that bacterial digestion of food precedes true gastric digestion. The action of the rumen micro-organisms on the food thus becomes of major importance in the nutrition of the older animal. The rumen in the young milk-fed animal at birth is but little developed, and the animal behaves nutritionally as having a simple stomach. In the older animal the rumen develops into an ideal site for microbial action; the saliva forms a buffer, keeping the contents about neutral in pH value, the numbers of organisms are kept relatively constant by passage to the abomasum and intestines, and nutrients are supplied at intervals by the animal feeding.

The capacity of the caecum and colon relative to the rest of the tract, and therefore the possible nutritional significance of the micro-organisms therein, varies with the species. The greatest capacity is found in non-ruminant herbivores such as the horse, where the size is such that the microbial digestion of bulky plant residues can take place. The large gut is relatively smaller in omnivores, but even so, in animals such as the pig, the rat, and man and other primates, the capacity is sufficient to make microbial digestion, on a significant scale, possible. Only in true carnivores is the relative size of the large gut so small that any microbial digestion that takes place is insignificant compared with the action of the gastric and small intestinal secretions.

II. THE ESTABLISHMENT OF THE MICROBIAL POPULATION IN THE ALIMENTARY TRACT

A. In Man

Witkowsky (1935), in an investigation of the origin of the mouth flora, found that the organisms could be demonstrated in infants shortly after birth and consisted mainly of *Staphylococcus albus*, coliform bacilli, Döderlein's bacillus and streptococci. Vaginal swabs from the mothers showed that these organisms were all present in the vagina in proportions corresponding fairly closely to those in the infant's mouth. Within a few days after birth other organisms appeared consisting in decreasing order of frequency, of pneumococci, haemolytic staphylococci, various types of streptococci, Friedländer bacilli, cornynebacteria, sarcina and Gram-negative cocci. Comparison with the mouth flora of the mother indicated that the infant might well have derived these additional organisms from the nasopharyngeal tract of its mother and other persons in its environment.

The intestine at birth is either sterile or contains only a few bacteria; infection rapidly occurs, partly from above and partly from below. Schild (1895) found that the anus became contaminated with organisms from the air, bath-water, and other objects. The meconium remains free of organisms for some 20 hr, by which time infection has occurred from the food.

The intestinal flora of the breast-fed infant consists mainly of *Lactobacillus bifidus*, and this may constitute 99 per cent of the total organisms in the faeces in the early weeks (Cruickshank, 1925). A few enterococci and coliform bacilli have also been reported (Tissier and Dreyfus, 1925). With weaning, the coliforms and enterococci increase and, at the same time, clostridia and members of the *Fusiformis* group become characteristic of the faecal flora, though *L. bifidus* may be found in some 25 per cent of weaned infants (Snyder, 1940). Staphylococci of both the *albus* and *aureus* species are often present, being probably derived from the infant's nose. But in the bottle-fed baby *L. bifidus* is uncommon and *L. acidophilus* is usually present in large numbers.

The unique presence of *L. bifidus* in the intestinal flora of breast-fed infants has stimulated research on the nutritive requirements of this organism (György, Norris and Rose, 1954). A particular variant, *L. bifidus var. Penn*, requires a growth factor that is present in human milk in 50 times the amount contained in cow's milk (György *et al.*, 1954). Tomarelli *et al.* (1954) have reported that this growth factor was

have been ingested with the food; but these, with the exception of acid-resistant vegetative bacilli and sporing bacteria, appear to be killed off rapidly.

There is general agreement that the normal jejunum and upper ileum are practically sterile, but in the lower ileum, where more alkaline conditions progressively increase, bacteria are often found, though in small numbers, and include viridans streptococci, *Streptococcus faecalis*, staphylococci, lactobacilli, *Clostridium welchii*, 'Veillonella' and occasionally *E. coli*.

These authors record that, in the normal large intestine, coliform bacilli of different types, enterococci, staphylococci of both the *aureus* and *albus* varieties, anaerobic spore-bearing organisms such as *Cl. welchii*, and *Cl. putrificum*, aciduric bacteria including *L. acidophilus* and *L. brevis*, thermophilic bacteria and yeasts are found. *E. coli* is predominant among the coliforms. The proportion of anaerobic to aerobic bacilli in the intestine, according to these authorities, has probably been underestimated by most workers. Anaerobic organisms are very common in the faeces. There is no doubt that considerable changes in the intestinal flora can be effected by the administration of sulphonamides and antibiotics. Aureomycin and terramycin in suitable dosage can virtually eliminate the normal aerobic bacteria of the gut, but resistant organisms such as yeasts, *Proteus*, *Pseudomonas*, and faecal streptococci may later take their place (Spaulding *et al.*, 1949; Di Caprio and Rantz, 1950; Bierman and Jawetz, 1951; Brisou and Ardisson, 1952).

The old conception that ill-health and actual well-defined disease processes could be due to the absorption from the intestine of toxic products elaborated by proteolytic micro-organisms, while plausible, still lacks experimental evidence (Topley and Wilson, 1955).

It is generally held that the replacement of a proteolytic by a saccharolytic flora–aciduric bacilli, *Cl. welchii*, and the enterococcus, can be largely accomplished by feeding on a high carbohydrate diet. By giving large quantities of lactose or dextrin in the diet, the intestinal flora can be so changed that it contains 90 per cent or more of aciduric bacilli (Rettger and Cheplin, 1921; Cannon and McNease, 1923; Cruickshank, 1928). These two carbohydrates are absorbed very slowly and consequently pass to the large intestine, where they are acted upon by aciduric bacilli—notably *L. acidophilus*—with the formation of a large amount of lactic acid: the presence of the acid is considered unfavourable to the persistence of proteolytic bacteria. Without the supply of lactose, dextrin or milk, the aciduric bacilli cannot be successfully implanted in the intestine, even though pure cultures are given daily in large quantity. According to Gerstley, Howell and Nagel (1932), 12 per cent of lactose must be added to whole cow's milk or to lactic acid milk in order to convert the mixed intestinal flora into a lactobacillary flora similar to that found in the stools of breast-fed babies.

From a consideration of the predominance of *L. bifidus* in the faeces of breast-fed but not artificially-fed infants, a chromatographic study was made by Ross (1950*a*) of the amino acids in the faeces of breast-fed and artificially-fed (dried milk) infants. In the breast-fed infants' faeces, alanine, glycine, glutamic acid, valine, and leucine were always present; serine, threonine, aspartic acid, and one or more of the lysine, ornithine, and arginine group were usually present. In the faeces of the artificially-fed infant only valine was present in all samples; glutamic acid, leucine, alanine, and glycine were frequently present, and occasionally traces of lysine and aspartic acid.

Of 75 infants admitted to hospital with a diagnosis of gastroenteritis, Ross (1950*b*) found that those showing a large proportion of Gram-negative organisms in their faecal smears were very ill.

B. In Ruminants

The rumen flora of the young ruminant differs from that of the adult animal. Normally, in the sucking calf or lamb the milk passes directly to the abomasum owing to reflex closure of the oesophageal groove, but in animals fed by artificial methods some of the milk can pass into the rumen. The rumen population of the young milk-fed animal is probably derived by the back flow of the contents of the abomasum which has been shown to occur after feeding (Trautmann and Schmidt, 1933). Gram-positive cocci, differing from the adult *Strep. bovis*, and lactobacilli are present in large numbers (Mann and Oxford, 1955), and coliform types, mainly *E. coli*, are also found in numbers of about 10^5/ml (Mackay and Oxford, 1954). Bryant *et al.* (1958*a*) have studied in detail the bacteria found in the rumens of milk-fed calves from 1 to 13 weeks of age. They found that counts of coliform types were highest in animals about 1 week old (*ca.* 10^9/ml). These workers also found high counts of lactate-utilizing organisms, and Hobson, Mann and Oxford (1958) have found a large Gram-negative lactate-utilizing coccus to be present in much higher numbers (10^{10}/ml) in the rumens of young calves than in older animals (10^4/ml). There are also many other bacteria present, and in calves reared under normal conditions the rumen population gradually approaches that of the adult as the animal grows older (Bryant *et al.*, 1958*a*).

It is very difficult to generalize about the state of the flora at any age, as the organisms present depend to a

also present in human saliva, salivary mucin, and hog gastric mucin, of which the latter was the richest source. A disaccharide has been obtained from hog gastric mucin which is some 50 times as active as a growth factor as the solids of skimmed human milk. It yields acetylglucosamine and galactose on hydrolysis and has been tentatively identified as galactose-acetylglucosaminide.

György *et al.* (1954) studied the distribution of this factor in a variety of natural materials, finding it to be present in large amounts in the colostrum of many species, in saliva, and in the meconium. By the use of chromatography, four different active components were obtained from human milk. All contained N-acetylglucosamine, and three contained fucose. In a more detailed study of an enzyme present in *L. bifidus var. Penn* Zilliken *et al.* (1954) found that this enzyme, when allowed to act on a mixture of lactose and N-acetylglucosamine, increased the biological activity of the mixture threefold. Two isomeric disaccharides were formed, one of which was identical with that isolated from hog mucosa. The addition to cow's milk of a human milk eluate, known to support the growth *in vitro* of this *L. bifidus*, did not increase nitrogen retention in infants although the pH of the stools decreased (Barness *et al.*, 1957).

Recently, lactulose, a keto-sugar prepared by the alkali treatment of lactose, has been claimed by Petuely (1957) as an effective promoter of *L. bifidus* flora in young infants.

The relatively high ratio lactose: protein in human milk has been singled out in the past as perhaps the most important factor in determining the bifidus flora in the breast-fed infant (Duncan, 1955). Lactose is not easily split by alimentary enzymic action in the intestine and the undigested portion may promote fermentation, the production of acids, and the propagation of aciduric lactobacilli in the large intestine. Lactose may thus improve the absorption of calcium. Johansson and Sarles (1949) claim that on a diet with lactose there is a greater synthesis of vitamins.

Strepogenin-like constituents of both human and cow's milk (György and Rose, 1955) and an additional enhancing factor, apparently specific for human milk (György, 1957), may act as supplementary growth factors, at least for the specific mutant of *L. bifidus*, namely *var. Pennsylvanicus*.

The role of *L. bifidus* in the physiology of the breast-fed infant is still shrouded in mystery, and not all would be prepared to go as far as Walch (1956) in suggesting that this organism is responsible for conferring immunity against certain enteric infections. This flora of the normal breast-fed infant is the pre-eminent example of a fermentative, saccharolytic, aciduric intestinal flora. The mixed flora of infants fed cow's milk, as well as the intestinal flora at all other ages, is characterized by a multitude of various fermentative and putrefying aerobic and anaerobic bacterial strains, with the prevalence of putrefaction. In 1949, Sears, Brownlee and Uchiyama recorded that the *Escherichia coli* flora of the intestinal tract of man at any one time consists usually of one or two strains which persist over long periods, together with a small number of other strains whose tenure is limited to a few days, a few weeks, or a few months. The long-tenure strains they designated as residents, the small number of other strains whose tenure is limited to a few days, a few weeks, or a few months were designated as transients. Further observations (Sears and Brownlee, 1952) demonstrated that this pattern of bowel *E. coli* may be shown by very young babies as well as by adults, resident strains becoming established as early as 10 days after birth. Diarrhoeic attacks of short duration do not by themselves usually bring about changes in the resident strains.

These findings, taken in conjunction with the modern concept of the importance of the pattern of the normal bacterial flora in the body economy, might suggest that the lactobacillary flora of the breast-fed infant is of intrinsic value. But it might well be argued that it would synthesize very different nutrients from those synthesized by the heterogeneous intestinal flora of the artificially-fed baby.

Although there is no really satisfying investigation concerning the localization of this flora in the intestinal tract of breast-fed infants, the investigations of Barbero *et al.* (1952), using double-lumen Miller–Abbott tubes, have permitted specimens to be withdrawn from various levels of the intestine. In general, the duodenum was relatively sterile, but bacteria, mainly enterococci, were found in the jejunum, and in increasing numbers towards the ileum. There was marked bacterial proliferation in the caecum and distal to it. The pH reached a peak of 7·0 to 8·0 in the caecum and tended to fall in the distal portion of the colon. This change toward the distinctly acid level was more pronounced in breast-fed infants than in infants fed cow's milk. *L. bifidus* was never cultured above the terminal ileum but was usually cultured from the large intestine of both breast-fed infants and infants fed cow's milk. The floral of the breast-fed infant consisted of over 90 per cent of Gram-positive bacilli, whereas that of the infant on cow's milk was variable. The feeding of brands of "adapted" milk failed to produce stools with an acidity and bacterial flora similar to those of breast-fed infants.

According to Topley and Wilson (1955) the adult empty stomach is generally sterile but, immediately after a meal, it contains numerous organisms which

large numbers of lactobacilli inhibits protozoal development in the calf (Eadie *et al.*, 1959). Counts of protozoa in rumen contents have varied widely between different workers, and also by the same workers on different occasions. This is due to the large size of the organisms, and the difficulty of obtaining representative samples from the rumen and from later dilutions of rumen contents. Boyne, Eadie and Raitt (1957), using a development of the method of Adam (1951), have assessed the magnitude of the errors in counting the protozoa and have found that day-to-day differences in numbers outweigh variations due to their experimental technique. These authors found that the total numbers of protozoa in a sheep on a concentrate and hay diet were of the order of 100 to 300×10^3/ml. These figures are similar to most other counts reported.

The metabolism of the holotrich protozoa has been studied in greatest detail, as it is possible to obtain massive suspensions of them relatively easily. The two genera represented, *Isotricha* and *Dasytricha*, are mainly concerned in the fermentation of soluble sugars, although the larger *Isotricha* will swallow and digest small starch granules, such as rice. It seems now to be established that the holotrich protozoa possess a considerable range of carbohydrases, independent of any enzymes provided by bacteria which they might ingest (Heald *et al.*, 1952; Heald and Oxford, 1953; Gutierrez, 1955; Howard, 1959; Mould and Thomas, 1958). The principal products of fermentation of glucose are acetic, butyric and lactic acids, carbon dioxide, hydrogen, and an intra-cellular amylopectin type polysaccharide. Gutierrez (with Hungate, 1957; 1958) has provided evidence to show that bacteria are ingested by the holotrich protozoa, and that the organisms are extremely selective in the bacteria which they ingest. For instance, *Isotricha prostoma* will select and ingest a number of Gram-negative rod-type bacteria from a mixed suspension, and these bacteria are stored in food vacuoles in the protozoa (Gutierrez, 1958). It is thought that these bacteria are a source of nitrogen for the protozoa.

The metabolism of the oligotrich protozoa has been little studied, since they cannot be obtained in washed suspensions as easily as the holotrichs, and only recently have they been cultured *in vitro*, then not in defined media. Microscopic studies have, however, shown that the larger oligotrichs will ingest plant particles and starchy and cellulosic materials. The smaller *Entodinium* spp. will ingest starch granules and may become almost the only protozoa in animals on a high starch diet (Van der Wath and Myburgh, 1941). Oxford (1958) has recently found that under certain feeding conditions (fresh red-clover diet) virtually the only oligotrich protozoa in the rumens of cows were of the species *Epidinium ecaudatum*. Crawley and he devised a method for obtaining and culturing these organisms *in vitro*. The protozoa ingested small starch granules from the clover leaves, and Bailey showed that the protozoal cells contained an α-amylase. The other carbohydrases present in the protozoa were maltase and cellobiase, the invertase and β-glucosidase found in holotrich protozoa being absent (Bailey, 1958). It would seem that this protozoan, like the holotrichs, possesses its own enzyme systems, as the organisms used in the above work were almost completely free from bacteria.

IV. BACTERIA IN THE ALIMENTARY TRACT

A. Bacteria of the Rumen

1. General Discussion

Alimentary bacteria can be beneficial to the host if they decompose otherwise indigestible materials such as cellulose, or if they synthesize molecules or specific groups which the host requires and which are absent in the diet, or present in a quantity insufficient to meet requirements. Both attributes are of benefit to the host only if the products of disintegration or synthesis can be absorbed from the alimentary tract. On this definition bacteria may be useful to the host if they ferment cellulose, pentosans or even raw tuber starch, which are otherwise indigestible, or if they synthesize members of the vitamin B complex, or amino acids when these are not present in the food in adequate amounts. This section will be devoted to a discussion of the metabolism of the bacteria present in the rumen.

Although direct microscopic observation of stained films and wet preparations continues to be of the greatest advantage in enumerating the main morphological groups of bacteria present in animals under different feeding régimes, and in observation of the types of bacteria attached to particulate foodstuffs, there are limitations set to this method by the pleomorphic character of many rumen bacteria, and by the possibility that many of the larger and more easily described bacteria are less important in the rumen functions than the smaller and more numerous ones. The development of media for the isolation of rumen bacteria, and the use of washed cell suspension and artificial rumen techniques, have led to a clearer conception of the role of many rumen organisms.

great extent on the type of ration being fed. For instance, Eadie, Hobson and Mann (1959) have shown that the numbers of lactobacilli and the large lactate-utilizing coccus are greatly influenced by the amounts of starchy concentrates in the ration. The presence of this large coccus and other lactate-fermenting bacteria, such as *Veillonella gazogenes*, may to some extent account for the occurrence of a larger proportion of propionic acid in the rumens of young animals than in older.

Hay and other roughages in the ration have been shown to be of importance in the development of the adult rumen population in calves (Pounden and Hibbs, 1948), but there is little evidence that, in calves reared under normal conditions, inoculation with rumen contents from adult animals has any influence on the development of the flora. If the correct ration is being fed the organisms will be picked up and develop naturally, and if the ration is unsuitable the organisms added as inoculum will not multiply. Since rumen protozoa have been shown to be transferred only by direct contact between animals (Becker and Hsuing 1929), calves reared in isolation generally fail to develop the normal fauna, and these animals have been described as "pot-bellied" and having rough coats, although their weights were similar to faunated animals (Pounden and Hibbs, 1948). The bacteria developing in isolated calves, up to 17 weeks old, have been studied by Bryant and Small (1956*a*), who found only a very slow development of the adult type flora compared with isolated, but inoculated, calves.

Flatt, Warner and Loosli (1958) have shown that the development of the rumen in calves is stimulated by the chemical products of the bacterial fermentations, *e.g.* volatile fatty acids, and not directly by the physical texture of the ration fed. McCandless and Dye (1950) have studied the decrease in blood sugar that occurs as the young ruminant develops and they suggest that the decrease in blood sugar is due to the relative increase in the quantity of short-chain fatty acids absorbed. McCarthy and Kesler (1956) have also found that the blood sugar levels decreased as the blood and rumen volatile fatty-acid levels increased. These transitions started at a week old, so that a calf is a partially functioning ruminant from this early age. Swanson and Harris (1958) found that rumination had begun in many calves by two weeks of age, and rumination time increased rapidly, as the animals grew older, to 5 hr per day at 6–8 weeks old. Rumination time was correlated with dry feed consumption, but less time was spent ruminating per pound of feed consumed as the calves aged.

C. In Birds

The caecal flora of the chicken develops extremely rapidly, and Lev and Briggs (1956*a*) have even demonstrated a flora, mainly clostridia and some *E. coli*, in the caeca of newly-hatched chicks that had not received food or water. A balanced intestinal flora is established by one day after feeding (Lev and Briggs, 1956*b*).

III. PROTOZOA IN THE ALIMENTARY TRACT

Various specialized kinds of ciliate protozoa are found in the rumens of cattle, sheep and goats, and in camels and reindeer. They have also been reported in a large number of wild ruminants. Other types have been noted in the caecum and colon of the horse, and according to Buisson (1923) various species are found in the large intestines of the African elephant, the rhinoceros, the American tapir, the guinea-pig, and the chimpanzee and gorilla.

Hungate (1955) has reviewed present knowledge of the flagellates of the wood-eating termites and *Cryptocercus punctulatus*. But only the rumen protozoa will be dicussed here.

A. Protozoa in Ruminants

There are two types of rumen protozoa, the oligotrichs and the holotrichs, but the different species vary in numbers with the diet of the animal. Oxford (1955) and Hungate (1955) have recently provided reviews about them.

For some time, doubt was cast on the utility of the protozoa in the general scheme of rumen metabolism, but recent investigations have shown that the protozoa can play a not inconsiderable part in the digestive processes occurring in the normal rumen, although they do not appear to be strictly essential, as bacteria can apparently perform all the metabolic processes carried out by the protozoa. Hungate (1955) has estimated that the mixed protozoa provide some 20 per cent of the protein requirements of the host. Gutierrez (1955) suggested, on the basis of his *in vitro* experiments, that the holotrich protozoa form some 10 per cent of the fatty acids in the rumen.

There is no evidence for encysted or free-living forms of the rumen protozoa, and the young animal must obtain its rumen fauna by contact with other animals (Becker and Hsuing, 1929). However, even if provided by inoculation, the protozoa will not develop unless the rumen contents are suitable for them. For instance, a low rumen pH caused by the presence of

1954; Hungate, 1957). Although very high numbers of these organisms grew in the rumen of a hay-fed sheep to which large amounts of grain were suddenly given (Hungate *et al.*, 1952), numbers do not increase when high grain rations are fed continuously (Higginbottom and Wheater, 1954; Hungate, 1957). Masson (1950, 1951) observed large numbers of *Cl. butyricum* in the rumen of a sheep fed a ration rich in flaked maize, but these do not appear to be of general significance in rumen starch digestion. Strains of *Strep. bovis* and *Cl. butyricum* have been shown to produce an amylase of the α-type (Hobson and MacPherson, 1952).

Although *Strep. bovis* is generally found in the rumen, other starch-fermenting organisms can equal or outnumber it. In rations low in starch and high in fibre Bryant *et al.* (1958*c*) found that a Gram-negative rod, *Bact. ruminicola*, formed a high proportion of the total amylolytic organisms, but rather strangely, with rations high in starch they formed only 10 per cent of the amylolytic bacteria. *Succinimonas amylolytica* is another amylolytic organism which generally forms a small proportion of the total starch hydrolysing bacteria, and *Butyrivibrio fibrisolvens* is usually amylolytic. Hamlin and Hungate (1956) found a very pleomorphic Gram-negative organism, *Bact. amylophilus*, in small numbers in the rumens of a number of cattle, and this was interesting in that it fermented only starch and maltose.

Warner (1956*a*) found that the ability of rumen contents to digest starch *in vitro* in an artificial rumen was greater if the contents were taken from an animal with starch in its diet. Even such relatively-resistant starches as potato are rapidly digested by bacteria which attach themselves to the granules in the rumen, and Weller and Gray (1954) found that the amount of starch reaching the small intestine was nutritionally insignificant, even when diets high in starch were being fed.

The end-products of fermentation of starch by different rumen bacteria are lower fatty acids, succinic and lactic acids, carbon dioxide and hydrogen.

4. Fermentation of Pentosans

Pentosans, of which the chief component is xylan, form some 16 to 20 per cent of the dry matter of grass and hay (Fraps, 1930; Hallsworth, 1939; Ekelund, 1949). Heald (1953) found that about 40 per cent of the xylan ingested by sheep was digested in the rumen, and he calculated that some 60–80 g of xylan may be fermented every 24 hours in the rumen of a sheep at pasture. Grey *et al.* (1958) have also found that a similar proportion of the hemi-celluloses in hay was digested in the rumen. Howard (1955, 1957), and later, Gray and Weller (1958), followed the degradation of pentosans by washed suspensions of mixed rumen bacteria. Mono- and oligo-saccharides were first formed and these were then fermented to lower fatty acids and CO_2.

It has been known for some time that the constituent sugars of pentosans, xylose and arabinose, are rapidly fermented by mixed rumen bacteria, and many rumen bacteria isolated in pure culture have been found to ferment xylose. However, fewer types have so far been shown to be active in fermentation of xylan. One xylan-fermenting type, which has been found to be widely distributed in the rumen contents of cattle, is *Butyrivibrio fibrisolvens*. A second xylan fermenter, isolated in high numbers from one sheep, is *Bact. amylogenes* (Doetsch *et al.*, 1957), and the enzymes of this bacterium have been studied in detail by Howard, Jones and Purdom (1958). They found that a cell-free extract contained at least three enzymes, one hydrolyzing xylobiose, xylotriose and xylotetraose but not pentosan, and two enzymes active in degrading the pentosans to "dextrins" and oligosaccharides. The end-products of fermentation of xylan or xylose by bacteria of the *Bact. amylogenes* type are mainly butyric acid with smaller amounts of other fatty acids, but as the end-products of the fermentation by mixed rumen bacteria are mainly acetic and propionic acids with little butyric (Gray and Weller, 1958), this suggests that other bacteria play a main part in digestion of xylans, or that subsidiary reactions occur. Although mixed rumen bacteria can ferment xylose, some xylan fermenting bacteria are unable to utilize the monosaccharide (Bryant *et al.*, 1958*b*). In this they are comparable with some of the cellulolytic bacteria, which can ferment cellulose and cellobiose, but not glucose.

5. Fermentation of Pectins

Leroy and Michaux (1949) found that sheep digested a high proportion of the "pectic substances" included in such foodstuffs as apple pulp, sugar-beet pulp, hay and straw. It was found that a sheep feeding on hay consumed from 75 to 102 g daily of pectic substances (Michaux, 1950). The proportion of pectic acid to pectin is high in apple and sugar-beet pulp, the ratio being approximately 1:10; the ratio of these substances in the faeces of sheep fed on these foods was 1:0·5. In hay, however, pectin predominates. The digestibility of pectic substances in sheep was approximately 75 per cent or over, for hay, and 90 per cent for apple and sugar-beet pulp, but in a lamb, the digestibility was considerably less (Michaux, 1951). Conrad *et al.* (1958) found by *in vitro* incubations that pectic substances increased gas production when added to washed alfalfa fibre. Hydrolyzed pectin produced the most marked effect.

Although some workers (Gall and Huhtanen, 1951) have postulated that a rumen bacterium must be a strict anaerobe, it has now been shown that, although this is often the case, there is, in the rumen of the sheep at least, a large population of facultative anaerobes all contributing towards the rumen function.

The number of bacteria in the rumen has been estimated by counts of wet preparations, in a counting chamber, and by dry film techniques. Both methods can give only approximate results because of the difficulties inherent in counting a population of organisms of mixed sizes, many of which are adhering to plant particles, but Moir and Williams (1950), using a counting chamber technique, have found numbers of free bacteria to vary from 25 to 64×10^9/ml rumen contents in sheep on different protein intakes. The number of bacteria in the rumen has been found to vary with time after feeding, and with the season of the year, even in sheep on a constant diet (Nottle, 1956). Various counts have been made of the total viable bacteria in the rumen, by dilution methods, and these have given figures comparable with the slide counts. However, no one medium is suitable for the growth of all rumen bacteria, and it would seem that viable counts give a better idea of the number of bacteria attacking a given substrate, rather than the total numbers.

As more bacteria are isolated from the rumen it becomes more difficult to point to any one organism as responsible for a particular reaction, and the following survey attempts to point out only the main types of bacteria which may be responsible for a given reaction.

2. Fermentation of Cellulose

The isolation of cellulose-fermenting bacteria from the rumen has received a considerable amount of attention, and a number of morphological types have now been isolated which can hydrolyse cellulose, usually in the form of cotton wool or powdered filter paper, in *in vitro* culture. Hungate (1950) isolated an actively cellulolytic Gram-negative rod, which he named *Bacteroides succinogenes*. This type of organism has since been shown to be generally active in rumen cellulose decomposition. Sijpesteijn (1951) also isolated a cellulolytic coccus, forming a yellow pigment, and named it *Ruminococcus flavifaciens*. Hungate (1957), however, found other types of cellulolytic cocci, named *Ruminococcus albus*, but there was considerable variation among them in properties and ability to digest cellulose. Bryant *et al.* (1958*b*) have made a survey of a large number of cellulolytic isolates of the genus *Ruminococcus*. Hungate (1957) also found spore-forming bacteria to be very active in *in vitro* degradation of cellulose, and these appear to be of importance in rumen cellulose digestion. The bacteria were identified as clostridia, of the species *Clostridium lockheadii* and *Cl. longisporum*. Some of the most common cellulolytic bacteria in the rumen seem to be Gram-negative rods, producing large amounts of butyric acid, and of the new genus *Butyrivibrio* (Hungate, 1950, 1957; Bryant and Small, 1956*b*; Gill and King, 1958). These formed 39 per cent of the cellulolytic isolates from 25 cows fed timothy hay, the cellulolytic cocci being 27 per cent of the isolates (Hungate, 1957). As well as these major isolations, some strains of other rumen bacteria have been found to be cellulolytic.

Owing to the general difficulty of their cultivation the enzyme systems of pure cultures of cellulolytic rumen bacteria have not been studied, but Halliwell (1957) and Festenstein (1958) have shown, by the use of whole rumen contents, that a number of enzymes are involved in the breakdown of whole cellulose. The ultimate products of the fermentation of cellulose by the rumen bacteria so far studied include short-chain fatty acids, carbon dioxide, hydrogen and lactic and succinic acids.

Cellulolysis by rumen micro-organisms *in vitro* is influenced by a number of factors. Hubbert, Cheng and Burroughs (1958) found that a number of inorganic ions were needed. Bentley *et al.* (1955) found that certain volatile fatty acids, mainly valeric, stimulated cellulose digestion by mixed rumen organisms *in vitro*, and Bryant and Doetsch (1954) showed that a mixture of a branched and straight chain fatty acids was needed as a growth factor by *Bact. succinogenes*. Later work (Allison, Bryant and Doetsch, 1958) showed that strains of *R. flavifaciens* also needed volatile fatty acid growth factors. Dehority *et al.* (1957) found that valine, proline, leucine and *iso*-leucine in protein hydrolysates were stimulatory to cellulose digestion by rumen micro-organisms, and in later work they showed that this was due to their conversion to volatile fatty acids by the rumen micro-organisms (Dehority *et al.*, 1958). Cline, Hershberger and Bentley (1958) also found that during cellulose digestion *in vitro* valeric acid disappeared, but during starch digestion it was produced. This suggested a reason for the increased cellulose digestion found when mixtures of starch and cellulose were digested *in vitro*.

3. Fermentation of Starch

In the early studies of Van der Wath and Myburgh (1941), coccoid bacteria were seen clustering around starch granules. Amylolytic streptococci of the *Strep. bovis* type have since been shown to be generally present in the rumen in numbers of about 10^9/ml (MacPherson, 1953; Higgin[illegible]

The bacteria responsible for the degradation of pectic substances in the rumen have not been studied in detail, but some strains of *Succinivibrio dextrinsolvens* and *Lachnospira multiparus* fermented pectin *in vitro* (Bryant and Small, 1956*c*).

6. Breakdown of Proteins

A number of experiments have now shown that a large proportion of the foodstuff protein of ruminants is broken down and resynthesized in the rumen into microbial protein. McDonald (1954, 1957) found that 40 per cent of zein and 90 per cent of casein in partially purified diets is so converted. Weller, Gray and Pilgrim (1958) have examined the rumen contents of sheep slaughtered at different times, after feeding a ration of wheaten hay, and found that, between 2 and 20 hours after feeding, microbial nitrogen accounted for 63 to 82 per cent of the total, soluble nitrogen 10 to 5 per cent, the rest being plant nitrogen. The rumen microbial nitrogen is of good digestibility (Reed, Moir and Underwood, 1949) and has an amino-acid content similar to that of good pasture protein (Holmes, Moir and Underwood, 1953; Weller, 1957). Weller (1957) reported that the protozoal protein is richer in essential amino acids, notably lysine, than the bacterial protein.

As intermediates in the breakdown of foodstuff protein and formation of microbial protein, peptides and amino acids are formed, and these are found in the rumen soon after feeding, but then disappear quite rapidly (Annison, 1956; Blackburn, private communication). Non-protein nitrogen of the foodstuff would be similarly utilized. Amino acids in rumen contents can be used either for microbial synthesis or be deaminated. The latter process is rapid in normal rumen contents, the final products being ammonia, carbon dioxide, volatile fatty acids and some lactic acid (Shazly, 1951; Lewis, 1955; Heuter *et al.*, 1958), as has been shown in *in vivo* and *in vitro* experiments. Unlike the proteolytic activity, deaminative activity in the rumen appears to depend on the protein in the diet, an easily hydrolysed protein giving a high deaminative activity. Among the fatty acids formed by deamination are some straight and branched chain ones which have been found to act as growth factors for rumen cellulolytic organisms (Bryant and Doetsch, 1954), and to increase cellulose digestion by mixed rumen organisms (Bentley *et al.*, 1955). The possibility that the "cellulolytic factor" activity of some amino acids is due to their conversion to fatty acids has already been mentioned (Dehority *et al.*, 1958).

On the whole, little is known about the deaminating bacteria. Some rumen strains have been found to produce ammonia when tested *in vitro*. Dohner and Cardon (1954) reported an interesting case of symbiosis between two rumen strains of *E. coli*. Pure cultures failed to ferment lysine, but the two together did so, to give ammonia, butyric and acetic acids. The rumen protozoa also appear to excrete ammonia (Warner, 1956).

Peptide nitrogen is probably used for bacterial cell synthesis (Warner, 1955), and some rumen bacteria (*e.g. Bact. ruminicola* and some Gram-positive rods Bryant *et al.*, 1958*c*; Huhtanen, 1955)) need growth factors which are found in protein hydrolysates, and which are probably peptides. Partial protein hydrolysates also increase the cellulolytic activity of rumen bacteria *in vitro* (Hall *et al.*, 1954) and it seems likely that peptides formed *in vivo* by hydrolysis of foodstuff protein play a similar role. The nitrogen requirements of rumen bacteria are, in general, quite simple, and large numbers will grow in media containing a small number of amino acids. Gilroy (1957) found that over 80 per cent of his strains of rumen bacteria would grow on single amino acids or ammonium sulphate as nitrogen source.

The breakdown of protein to form these products has been studied in more detail recently, but the actual agents of proteolysis have not been entirely enumerated. The protozoa appear to be responsible for a large proportion of the rumen proteolytic activity (Warner, 1956*b*; Hunt, 1957; Blackburn and Hobson, unpublished), and proteolysis by the bacteria appears to be caused by organisms of all sizes (Hunt, 1957; Blackburn and Hobson, unpublished). However, the number of proteolytic bacteria so far isolated and characterized is comparatively small, and a large proportion are facultative anaerobes. Appleby (1955), studying three sheep, reported that bacilli, mainly *Bacillus licheniformis*, were the most numerous proteolytic bacteria present, but this finding has not been substantiated (Gilroy, 1957). Other proteolytic bacteria identified have been streptococci, micrococci, flavobacteria, chromobacteria, propionibacteria and occasional clostridia (Gilroy, 1957; Hunt, 1957; Blackburn and Hobson, unpublished; Appleby, 1955), and others have not yet been identified. Bryant and Small (1956*b*) found that *Butyrivibrio fibrisolvens*, which is common in the cattle rumen, is proteolytic, and Gilroy (1957) found that some of the proteolytic bacteria that he isolated were probably strains of this organism.

Although the large rumen bacteria appear to be proteolytic (Hunt, 1957; Blackburn and Hobson, unpublished), Bryant (1956) found that only 1 out of 11 strains of *Selemonas ruminantium*, a common member of the larger bacteria, hydrolyzed casein.

The degradation of protein in the rumen is thus a complex process, and the proportions of the final

caecal flora of hens was extremely varied in types, but no cultural studies were made. The observations of Shrimpton (1958) indicate that the type of caecal fermentation probably depends on the nature of the diet, for example, little methane was produced when maize was a major constituent.

The pH of the caecal contents changes from the tip, where it is highest, through the length of the caecum to the caecal duct, where it is lowest, so it is unlikely that these contents are homogeneous. It would seem as if the caecum contains, at any one time, regions where the bacterial fermentation is proceeding from a relatively long established microflora, and others, possibly near to the caecal duct, where the fermentation results from a microflora only freshly introduced from the small or large intestine.

The study of the microflora of the caecum is greatly facilitated by the establishment of a permanent fistula (Beattie and Shrimpton, 1957). Beattie and Shrimpton (1958) are in agreement with Sunde *et al.* (1950), who observed that in spite of the presence of microbiological activity within the caeca of the normal bird, caecectomized birds grow at least as well as controls. But it cannot be concluded that the caecal microflora has no effect upon the birds, but only that, under these standardized conditions, it had no net effect. There may be advantage under some untoward conditions.

E. Fermentation in the Rabbit Stomach

The rabbit is a herbivore in which development of both stomach and large intestine would seem to have taken place for the digestion of cellulose and related substances. But since the rabbit stomach epithelium secretes an acid juice, it does not seem likely that the interior of that organ would be a suitable microbial fermentation chamber. Moreover, Elsden *et al.* (1946) found very little volatile fatty acid in the rabbit's stomach compared to that contained in the caecum. However, the experiments of Stern, Hukovic and Fukarck (1955), which showed that the digestion of starch by rabbits depended on the activities of micro-organisms, and those of Herndon and Hove (1955), who found the digestion of cellulose in normal and caecectomized rabbits was the same although the colons of the latter animals were enlarged, suggested that some fermentation might take place in the stomach. It was, nevertheless, with surprise that Alexander and Chowdhury (1958) found that lactic acid was produced at such a low pH. The lactic acid produced in the stomach appeared, in part, to be absorbed through the gastric epithelium, also in the first part of the small intestine. The nutritional implications of coprophagy have still to be determined, but the gastric mucosa would be an area of absorption for products of micro-organismal activity.

V. INTERMEDIARY METABOLISM WITHIN THE RUMEN

The main function of the micro-organisms of the rumen is the digestion of feedstuff carbohydrates and proteins. The end-products of these primary reactions are, however, not always apparent in the rumen. For instance, although, as was mentioned above, lactic and succinic acids are produced by large numbers of rumen bacteria during fermentation of carbohydrates *in vitro*, little of these acids is normally found in the rumen. Again, hydrogen is a common end-product of bacterial fermentations, but a negligible amount is found in the rumen gases. These results suggest that many of the primary bacterial products are themselves fermented, to give, in some cases, products of value to the host animal, and in other cases, compounds which are wasted as far as the metabolism of the host animal is concerned.

In the first group are the acids lactic and succinic. Lactate can be absorbed from the rumen, but a larger proportion is metabolized to short-chain fatty acids, principally propionic (Heuter *et al.*, 1956). A large Gram-negative coccus has already been mentioned as one of the organisms bringing about this transformation in the young animal. While a number of lactate-utilizing bacteria have been isolated from adult sheep and cattle, two only have been studied in detail, *Propionibacterium acnes* (Gutierrez, 1953) and *Veillonella gazogenes* (Johns, 1951). These two organisms metabolize lactate by way of succinate, and so may also be concerned in the metabolism of succinic acid in the rumen.

Besides reactions such as these, in which fermentation products are further metabolized, the complete degradation of polysaccharides may involve more than one bacterial species, and it is quite possible that the simpler sugar products of hydrolysis of polysaccharides are liberated into the rumen liquid, where they are available to all the organisms. Experiments have shown that there are more organisms which will utilize simple sugars than are able to utilize the more complex carbohydrates, and in many diets these sugars would be lacking, unless supplied by the breakdown of more complex materials.

The metabolism of hydrogen and of formic acid form an example of the second type of intermediary metabolism, namely, that leading to products of little or no use in the metabolic processes of the host

rumen by microbial action (Reiser and Roddy, 1956; Shorland *et al.*, 1957). Recent experiments of Garton, Hobson and Lough (1958) have shown that glycerides are rapidly hydrolyzed by rumen microbial action both *in vitro* and *in vivo*, and so that, unlike single-chambered stomach animals, most of the dietary lipids reach the small intestine as free fatty acids. There is as yet little data on the micro-organisms responsible for this lipolytic action of the rumen contents. The glycerol liberated by hydrolysis of dietary lipids is fermented in the rumen to fatty acids, probably mainly propionic (Johns, 1953).

9. Nitrate and Sulphate Metabolism

Nitrate is reduced by rumen contents with the formation of nitrite. It has been calculated that 10 kg of dry plant material, which can be consumed by a cow in a day, could contain 620 g of nitrate (Holtenius, 1957), so quite large amounts of nitrate may be consumed by animals at pasture, and under certain circumstances it is possible for symptoms of nitrite poisoning to be produced, for instance, in oat hay poisoning. This might be cited as one of the less desirable effects of a predominantly microbial digestive system. However, rumen contents will also decompose nitrite, and under normal conditions, in animals on an adequate diet, nitrite is only transitorily formed. The ultimate decomposition product of nitrate is probably mainly ammonia, and this could be utilized in the usual way, but other reactions could be brought about by the rumen bacteria, and these have been reviewed by Holtenius (1957).

A source of sulphur is necessary for protein synthesis from added or endogenous ammonia in the rumen, and this can be supplied in the form of sulphate. ^{35}S from sulphate is incorporated into the bacterial protein *in vitro* and *in vivo* (Müller and Krampitz, 1955; Block, Stebol and Loosli, 1951). Sulphate is rapidly reduced to sulphide in the rumen (Lewis, 1954). In addition to incorporation of sulphur into bacterial protein, sulphur-containing amino acids can be broken down to produce sulphides. Sulphides can assist in maintaining the anaerobic conditions necessary for growth of many of the bacteria in the rumen, and may be necessary for growth of some of them, but an excess production of hydrogen sulphide in the rumen could have a deleterious effect on the well-being of the host animal.

B. The Alimentary Bacteria of Pigs

Willingale and Briggs (1955), also Raibaud *et al.* (1957), have found that streptococci of Group D, and lactobacilli, mainly *Lactobacillus acidophilus* and *L. fermenti*, are the most common bacteria in the intestinal tract of the adult pig. Coliform bacteria, and some other types such as micrococci and bacilli, are also present. Michel (1957) has studied the deaminations and other reactions occurring in the intestines of pigs and related these activities to different groups of bacteria. He found that the maximum deaminative activity of the intestines was in the caecum. Ammonia was present both in the intestines and in the portal blood. A large amount of amino acids was liberated by autolysis of the caecal bacteria, but the nutritional significance of this was not determined. The whole intestinal flora has a feeble proteolytic and amylolytic activity. Vitamin B_{12} was synthesized in the caecum, and choline was rapidly destroyed by the intestinal flora. Trautmann and Asher (1942) showed the presence of cellulolytic bacteria in the caecum of the pig, and Baker *et al.* (1950) found *Clostridium butyricum* to be an amylolytic bacterium in the caecum.

C. Bacteria in the Large Intestine of the Horse

As mentioned earlier, the large intestine of the horse is adapted for the fermentation of plant residues which are unattacked by the normal digestive processes. Little systematic investigation has been made of the microbial digestion occurring here, but from results so far obtained, reactions similar to those in the rumen seem to take place. Elsden *et al.* (1946) showed that the volatile fatty acids, acetic, propionic and butyric, occurred in the caecum and colon of the horse in amounts similar to those in the rumen. Alexander (1952) found that the concentrations of volatile fatty acids varied with the diet and with time after feeding. The difference in volatile fatty-acid production in a horse fed on hay or grass was not as great as the difference in rumen volatile acids of a sheep fed on these two materials. This was assumed to be due to the more soluble carbohydrates of the grass being utilized before they were subjected to microbial action in the horse's large intestine. In the sheep they would be available to the rumen microorganisms. Cotton thread was digested in the caecum and colon of the horse at a similar rate to that found in the rumen. Alexander, MacPherson and Oxford (1952) isolated Gram-positive streptococci, similar to *Streptococcus bovis*, and *Veillonella gazogenes* from the horse colon.

D. Bacteria in the Bird Caecum

The major microbiological activity of the caecum of the bird is fermentative rather than putrefactive, since the principal gaseous products of metabolism are methane and carbon dioxide, hydrogen sulphide and ammonia being minor constituents. Masson (1954) found by microscopic observation that the

stain satisfactorily, and have the appearance of dying cultures (Masson, 1950), which suggests changes in the cell walls. Extensive cultural work on the abomasum and intestinal contents would be needed to obtain a complete picture of the fate of rumen micro-organisms. A limited series of observations on a sheep with cannulae in the small intestine has suggested that there are few bacteria, other than sporing types, in a viable state in the proximal part of the small intestine, but that the numbers increase as the pH rises along the intestine, and in the ileum there is a more varied flora, with Gram-positive cocci and Gram-negative rods (the latter able to grow on MacConkey medium) becoming numerous (Blackburn and Hobson, unpublished). Since, however, a large part of the rumen protein is formed by micro-organism cells some, at least, of this microbial protein must be utilized by the animal. Hogan has shown that about 64 per cent of the total digestible nitrogen of the food is absorbed from the small intestine, but no determinations of the form that this nitrogen takes were made (Hogan, 1957).

Soluble carbohydrates are rapidly fermented by rumen organisms, and insoluble carbohydrates can be fermented to a large extent. For example, Gray *et al.* (1958) found that up to 50 per cent of the cellulose of the diet in sheep fed only on different hays or hay and straw was digested in the rumen. Weller and Gray (1954) suggested that the amount of starch escaping digestion in the rumen and passing to the small intestine was nutritionally insignificant, even in animals on high starch diets. A large proportion of the energy of the animal must therefore arise from the ruminal fermentations, or absorption of sugars from the stomach compartments. The blood concentration of glucose in ruminants is small, and feeding of glucose to a sheep did not result in any appreciable increase in the blood glucose levels (Schambye, 1951*a*, *b*). It is difficult to get a measure of the fatty acids absorbed from the rumen in animals on different diets, but Carroll and Hungate (1954) have calculated that the amounts of acids absorbed from the rumens of cattle on hay, or hay plus grain rations, represent about 70 per cent of the total energy requirements of the animals. However, the position is not entirely clear as Emery, Smith and Huffman (1956) think that the short-chain fatty acids provide only 3 to 13 per cent of the energy requirements of a cow. Armstrong and Blaxter and co-workers (1957*a*, *b*; 1959) have recently published data on the utilization by sheep of the energy in lower fatty acids administered, either separately or as mixtures, into the rumen. Although the utilization varies with the nutritional state of the animal, the fatty acids can provide a considerable proportion of the energy requirements of the animals. However, numbers of experiments have established that lower fatty acids, principally acetic, are present in the blood of cattle and sheep, and that the concentration varies with the concentration in the rumen (Reid, 1950; McClymont, 1951*a*, *b*). The metabolic pathways of the rumen fatty acids are not completely elucidated. Propionate is a precursor of carbohydrate; the fate of butyrate is more controversial, and although milk fats can be synthesized from acetic acid the other aspects of its metabolism are unsettled.

Similar mixtures of acetic, propionic and butyric acids are found wherever microbial digestion occurs, for instance, in the large intestine of animals including ruminants.

The synthesis of vitamins by micro-organisms of the rumen has been reviewed by Kon and Porter (1954). Gall and Huhtanen (1951) have isolated bacteria, Gram-positive anaerobic rods, from the rumen capable of synthesizing members of the vitamin B group. Besides being available to the host animal vitamins synthesized by some bacteria might be growth factors for other bacteria concerned in essential rumen metabolic activities, and in this connexion Lev has recently shown that a rumen strain of *Fusiformis nigresens* needed for growth vitamin K supplied by another rumen bacterium (Lev, 1958). Further discussion will be found in the next section where the synthesis of vitamins by intestinal bacteria is reviewed.

B. Synthesis of Vitamins

Cohendy (1912) concluded, from his work on chicks reared in a sterile fashion, that life is possible with a sterile intestinal tract, but that the intestinal bacteria are probably of use in assisting digestion. In 1923, Scheunert and Schieblich showed that *Bacillus vulgatus* living in the intestines of herbivores is capable of synthesizing vitamin B_1. Somewhat later, McElroy and Gross (1930, 1939) demonstrated that considerable amounts of riboflavin, pyridoxine and the anti-haemorrhagic vitamin are formed in the rumina of sheep and cows fed on diets low in these vitamins, and the source was assumed to be the commensal micro-organisms. Almquist, Pentler and Mecchi (1938) demonstrated that *Bacillus subtilis* and *Escherichia coli* could synthesize vitamin K. The synthesis of vitamins in substantial amounts, as in the reticulo-rumen, is not a peculiarity of its bacteria, for *in vitro* the intestinal bacteria of man, for instance *E. coli* and *Aerobacter aerogenes* synthesize biotin, riboflavin, thiamine, nicotinic acid (Burkholder and McVeigh, 1942) and vitamin K (Dam *et al.*, 1941).

The role of the intestinal flora as a source of vitamins, synthesized by bacteria, was discussed as early as 1914 by Cooper. The first most convincing

animal. Methane constitutes some 30 per cent of the rumen gases, carbon dioxide accounting for all but 1 or 2 per cent of the remainder (Lugg, 1938), and one of the sources of methane is hydrogen and carbon dioxide. Hydrogen is metabolized extremely rapidly by rumen micro-organisms (McNeill and Jacobson, 1955), and any formed by bacterial fermentations would have only a transitory existence. Methane is also formed by fermentation of formate, which again would have only a limited existence in normal rumen contents (Carroll and Hungate, 1955). Smith and Hungate (1958) have recently isolated in pure culture, one bacterial species which produces methane from formate, but as methanobacteria, in general, need very exacting conditions for growth, most of the information on methane production has been obtained by experiments on mixed rumen organisms. Nelson *et al.* have shown that acetate, butyrate and valerate can be intermediates in rumen methane formation (Opperman *et al.*, 1957; Nelson, Opperman and Brown, 1958).

The further reactions of the products of protein hydrolysis were dealt with earlier.

The primary products of lipolysis of dietary fats are the higher fatty acids and glycerol, and glycerol is rapidly fermented by rumen contents to give propionic acid and probably other, at present undetermined, products.

Among the other products of microbial metabolism are the bacterial and protozoal polysaccharides. Large numbers of rumen bacteria are encapsulated (Hobson and MacPherson, 1953) and some of these capsular polysaccharides are complex in composition (Hobson and MacPherson, 1954; Oxford and Bailey, 1949). It seems unlikely that these materials can be further metabolized in the rumen or in the intestines, but they can be a source of digestive disturbances in the animal. For instance, it has been suggested that bacterial carbohydrates of a "gummy" or "slimy" nature can act as stabilizing agents for the foams produced in the rumen during cases of "frothy" bloat. Some of the bacteria form starch-type intracellular polysaccharides. In some cases it seems that these polysaccharides are formed in dying cells, and so are not further metabolized (Hobson and Mann, 1955), but in other cases the polysaccharides are deposited during active fermentation of external carbohydrate, and are then metabolized by the bacteria when the external carbohydrate has been utilized (Howard, 1955; Doetsch *et al.*, 1957). The protozoal starch, which is rapidly deposited while the organisms are digesting foodstuff carbohydrate, is also in this latter category (Oxford, 1951). This type of metabolism is probably of benefit to the host animal as it allows of a more gradual release of fatty acids into the rumen contents than would be the case if all the foodstuff carbohydrate were immediately fermented to acids.

Besides the examples mentioned above, there are probably other interconversions occurring among the rumen fermentation products. For instance, rumen contents will utilize acetic and propionic acids *in vitro* with the formation of higher fatty acids (Gray *et al.*, 1952), and mixed rumen bacteria will metabolize carbon dioxide. Otagaki *et al.* (1955) found that when $^{14}CO_2$ was incubated with rumen contents, then ^{14}C appeared in the volatile acids and the protein of the micro-organisms. Huhtanen *et al.* (1954), using growing pure cultures of rumen bacteria, showed that ^{14}C from $Na_2{}^{14}CO_3$ was incorporated to varying extents into the bacterial cells.

The utilization of valeric acid, produced by starch fermentation, during cellulose digestion by rumen organisms has already been mentioned.

VI. THE UTILIZATION OF THE MICRO-ORGANISMS AND PRODUCTS OF MICROBIAL ACTION

A. General Discussion on Ruminants

Since extensive breakdown of the foodstuffs takes place in the rumen, it follows that the ruminant is largely dependent on the products of microbial action for its supply of nutrients. These can become available to the host animal either by absorption from the rumen, or by passage to and absorption from the intestines either with, or without, further enzymatic action. There does not appear to be any absorption of amino acids from the rumen, and while ammonia is absorbed it is then largely lost to the animal by secretion as urea in the urine. The only pathway of utilization of microbial nitrogenous products must thus be by absorption from the intestine. Rumen microbial protein is of good amino acid content and, when dried, it has a high value as a protein for non-ruminants such as rats. It is thus assumed that it will be equally valuable to the ruminant. The larger rumen bacteria and the protozoa visibly disintegrate in the abomasum and proximal part of the small intestine, but the smaller bacteria are more resistant. The microscopical studies of Pounden, Ferguson and Hibbs (1950) suggest that rumen bacteria vary in their resistance to digestion in the intestines. However, even if the bacteria do not completely lose their morphology, it is possible that changes in the cell walls allow of the solution and digestion of some cell constituents. The bacteria in the abomasum do not

vitamin may originate from the intestinal flora, but even more important is the recognition of the possibility that, under disturbed conditions, the supply of an unknown factor which is normally produced by the flora is reduced to a point where a conditioned deficiency develops.

1. Cobalt Deficiency and Vitamin B_{12} in Ruminants

Smith and Loosli (1957) state that with high-level cobalt injections, enough of the element reaches the rumen to stimulate a slow response in the cobalt-deficient sheep. How much cobalt reaches the rumen is not clear. The fact remains, however, that 1 mg per day of cobalt, when given by mouth to lambs, elicits a good response but will not do so when given parenterally, and thus attention was focused on the digestive tract as the probable area of cobalt need. This is the reticulo-rumen. That the lower tract is not involved is derived from knowledge that bile is a significant, though not the major, path of excretion. Further, Phillipson and Mitchell (1952) found that 0·1 mg of cobalt which is effective in sheep when fed, is not effective when introduced directly into the duodenum. Quantities of cobalt much higher than 0·1 mg daily, when injected into the duodenum or abomasum, did result in positive response in cobalt-deficient lambs and were correlated with increased cobalt concentrations in rumen digesta, most probably as the result of antiperistalsis.

Given parenterally cobalt produced no significant increase of vitamin B_{12} in the blood or body tissues of sheep but caused an increase of it in the contents of the caecum and large intestine, which was attributed to bacterial synthesis stimulated by excretion of cobalt into the intestine in the bile (Kercher and Smith, 1956).

Cobalt-deficient lambs respond quickly to vitamin B_{12} therapy if given enough, *e.g.* 300 μg over a two-week period. Cobalt deficiency, according to Smith and Loosli, is essentially a deficiency of vitamin B_{12}. The work of Kercher and Smith (1955) suggested that only some 3 per cent of the orally-administered B_{12} is absorbed. Cobalt supplementation is much the cheaper method.

There is a positive correlation between the cobalt intake and the vitamin B_{12} concentration of rumen contents of sheep (Hale *et al.*, 1950). The faeces are even richer than rumen contents.

As to recent evaluations of cobalt requirement, Marston (1952) holds that this is, for the sheep, of the order of 0·07 to 0·08 mg cobalt daily, and is furnished by a ration containing 0·08 to 0·10 p.p.m. cobalt on a dry-weight basis. Earlier New Zealand studies suggested that cattle may have a lower requirement than sheep. It was estimated that pastures containing 0·04 p.p.m. on a dry-weight basis were borderline for cattle. In the absence of better data Smith and Loosli (1957) consider that cobalt intakes adequate for sheep should be adequate for cattle. The soluble salts: sulphate, chloride or nitrate are used for therapy.

2. Strict Vegetarianism in Man

Wokes, Badenoch and Sinclair (1955) investigated a group of strict vegetarians, called "vegans," in Great Britain, and found that a high percentage of them exhibited B_{12} deficiency, as indicated by low serum B_{12} levels and by neurological symptoms. The serum B_{12} levels among the group were the only clear indications of B_{12} deficiency, ranging from 45 to 193 μμg per ml compared to 200 to 320 for normals. There was little anaemia, probably because the diet was high in folic acid.

Although vitamin B_{12} is present in the large intestine as a result of bacterial synthesis, Hausmann, Ludwig and Mulli (1953) have provided evidence that this is not directly absorbed by the body. This indicates that the chief source of the vitamin is dietary in nature and, since vitamin B_{12} is not present in plant material, it would appear that vegetarians who exclude animal food from their diet should develop a deficiency. Pollycove, Apt and Colbert (1955) studied a man of 60 who had avoided all foods of animal origin for eight years. This individual had a serum vitamin B_{12} level of 0·07 μμg per ml and a macrocytic anaemia which responded to parenterally administered vitamin B_{12}. By studies with ^{60}Co vitamin B_{12} it was shown that absorption from the gastro-intestinal tract was within normal limits.

C. Factors Affecting Absorption

There is ample evidence that, in man, absorption defects can also produce a vitamin-B_{12} deficiency (Ford and Hutner, 1955). The absorption of vitamin B_{12} when ingested in physiological amounts is dependent upon the presence of an intrinsic factor elaborated by the gastric mucosa. In pernicious anaemia, and in total gastrectomy, the absorption of vitamin B_{12} does not occur unless an intrinsic factor source is given, or relatively large amounts of the vitamin are administered so that a small portion diffuses across the intestinal barrier.

Nyberg and Östling (1956) have examined the levels of serum vitamin B_{12} in patients with fish tapeworm (*Diphyllobothrium latum*) anaemia. The average value found was about 50μμg per ml while the controls averaged 561 μμg per ml. No overlap in values was found. While these results support the long-held belief that the fish tapeworm somehow makes unavailable the B_{12} of the diet, they do not indicate whether this

proof for the biosynthetic ability of the intestinal flora for a vitamin in man was furnished by the study of the prothrombin levels as an index of vitamin K activity in the newborn infant (Dam *et al.*, 1942).

Luckey *et al.* (1955*a*, *b*) and Luckey (1956) have reviewed the work on vitamin metabolism in germ-free animals. The nutritional requirements of such animals are qualitatively the same as those for the conventional. Studies on the metabolism of germ-free rats with respect to food and water intake, growth rate, and reproduction, led to the statement: "It is possible that the net result of (microbial) intestinal synthesis may be disregarded as a nutritional factor." In a complete vitamin balance study run on germ-free rats fed a synthetic diet, three different states of vitamin metabolism could be seen. In one, less vitamin was recovered in the excreta, waste food, lost hair, and carcass than was put in with food. With a small group of vitamins, including pantothenate and vitamin B_{12}, the balance between intake and output was the same. In the third group, exemplified by vitamin B_6, more of the vitamin was recovered than was fed, indicating a net synthesis.

How far this is true of man is unknown.

Attention has been closely focused upon the utilization of microbially-synthesized vitamins ever since the demonstration of rice starch refection in rats, almost simultaneously, by Fridericia (1926) and Roscoe (1928), and of potato starch refection by Kon (1945). Around this topic has grown a vast literature. It must suffice to note that the evidence presented by Baker *et al.* (1950) for the pig, and by Masson (1954) for the chick, make it almost certain that the micro-organisms responsible for the synthesis of the B-complex are also the agents directly concerned with the breakdown of starch in the caecum. For the pig, raw potato starch, because of its less efficient digestion in the small intestine, constitutes a larger source of substrate for the micro-organisms of the caecum and colon, whereas cooked starch, though of higher digestibility in the small intestine, and hence of more value as a potential source of energy, is nevertheless less available as a source of substrate, and in feeding situations where there is a deficiency of B-vitamins the feeding of raw starch protects against symptoms arising from B-deficiency.

Ferrando and Lallouette (1958) found, in pigs, that the growth stimulation factor for coliform bacteria, as observed in the faeces and derived from *Aspergillus flavus*, was suppressed by copper sulphate and by molasses.

Phillipson and Reid (1957) found, in sheep, that the thiamine values were highest in the duodenum and low in ileum, caecum and faeces. In sheep fed on hay alone, there was some increase of the vitamin in the caecum, especially in solid matter, which suggests bacterial synthesis. Synthesis *in vivo* has been demonstrated in two ways, namely, by noting the continued excretion of a vitamin in an animal upon a vitamin-free diet—depletion of the body reserves being excluded as a possible source—and by the induction of avitaminosis by partial sterilization of the intestinal contents with sulphaguanidine or succinyl sulphathiazole, followed by the cure of the avitaminosis, during drug therapy, by giving adequate doses of the appropriate vitamin. According to Gant *et al.* (1943) the sulphonamide may act through reducing the number of vitamin-synthetizing bacteria, or by reducing their ability for synthesis.

Copping and White (1957) found, in rats, that when the diet contained glucose as the source of carbohydrate, coli-aerogenes organisms appeared to be more numerous in both caecum and rectum of pyridoxine-deficient rats than in those of rats receiving adequate pyridoxine, but when the glucose was replaced by starch no difference could be detected. Bacteria isolated from human faeces and cultured, formed riboflavin *in vitro*. Some of this was present outside the cells (Morishita, 1957). Production of extrabacterial riboflavin was greatest in cultures of *Alcaligenes faecalis* and least in those of enterococcus and staphylococcus. Production of intrabacterial riboflavin was also greatest in *A. faecalis* and least in *Proteus vulgaris*.

In the alimentary tract of the rat, not only vitamin K (Black *et al.*, 1942), biotin, folic acid (Nielsen and Elvehjem, 1942), the B-complex, and vitamin E (Daft and Sebrell, 1942), but also, possibly, certain essential amino acids (Martin, 1944) are synthesized by bacteria and used by the host. In man, a similar dependence upon intestinally-synthesized thiamine (Najjar and Holt, 1943), riboflavin (Najjar *et al.*, 1944), and nicotinic acid (Ellinger, Coulson and Benesch, 1944) has been demonstrated.

In studies on adult subjects kept on a restricted diet for 15 weeks, without the use of sulphonamides or antibiotics, faecal excretion of all vitamins studied, except niacin and pyridoxine, exceeded intake (Denko *et al.*, 1946). The combined urinary and faecal excretion of all vitamins studied, except pyridoxine, approximated to, or greatly exceeded, intake on the restricted diet. Urinary excretion of biotin and pantothenic acid approximated, and in several instances exceeded, intake on the restricted diet. Many vitamins may be synthesized in the intestinal lumen of man by microbial action, or are excreted into it through the intestinal wall.

Elvehjem (1948) has pointed out that not only is it important to know that under optimum conditions a significant fraction of the total requirement of a known

(Jukes, 1951), support the view that the effect is on the intestinal microflora. Since the intestinal flora had previously been shown to synthesize vitamins, it was natural to inquire into the effect of antibiotics on vitamin synthesis. Evidence for a sparing effect was obtained (Stokstad, 1955).

On human subjects, only a few observations on the action of antibiotics in hastening growth have been carried out. The most impressive positive results with antibiotics on growth of children are those reported by Schrimshaw and Guzman (1953) and Schrimshaw, Guzman and Tandon (1954), on Mayan children 7 to 12 years of age living in the Guatemalan Highlands and subsisting on diets low in animal protein. A pronounced growth effect followed the administration of chlortetracycline. The response with penicillin was variable. A slight but definite effect of chlortetracycline upon the rate of weight increase, but not on the rate of height increase, was observed in a study on school children in Jamaica with a mild degree of malnutrition (Mackay *et al.*, 1956).

Waisman and Boldt (1957) found that chloromycetin, dihydrostreptomycin, bacitracin and polymyxin were without effect on the growth of male rats on a tryptophan-deficient diet, and penicillin had a doubtful effect. Terramycin, achromycin and erythromycin significantly increased the growth of male rats but were less effective with females. There was no significant difference among growth rates when tryptophan was given alone or with an antibiotic.

Wiseman *et al.* (1956) agree with the observations of Rhodes *et al.* (1954) that antibiotics may depress the vitamin-requiring bacteria and increase the vitamin synthesizing micro-organisms.

Michel and François (1955) have studied the effect of antibiotics on the deamination of amino acids by the intestinal microflora of the pig. They found a relation between the index of growth and the capacity of the antibiotic to inhibit deamination of arginine. They also showed that copper and 3-nitro-4-phenylarsenic acid also possess this capacity to inhibit deamination. There is an accompanying diminution in the level of ammonia in the blood.

1. Water-soluble Vitamins

In gastrectomized rats 90 days after operation the vitamin B_{12} content of the liver was low compared with that of intact rats. The moderate anaemia was not corrected in these gastrectomized animals by vitamin B_{12} by mouth or by injection, and vitamin B_{12} given with folic acid or a pig stomach preparation had no effect in preventing the development of anaemia. When gastrectomized rats were given a supplement of aureomycin, haemoglobin values were within the normal range, but there was no apparent increase in the vitamin B_{12} stores in liver (Swendseid, Long and Halstead, 1957). But it has to be noted that while a vitamin-sparing action can, in part, explain the growth response on diets marginal in vitamins, it does not account for the growth observed in nutritionally complete rations. It must be noted that the ability to get a growth response with either an antibiotic or a vitamin on a suboptimal diet does not establish a vitamin-sparing effect. The only true criterion is a reduction in the vitamin requirement, as measured by a dosage response curve, or a greater response to an antibiotic on a suboptimal level than on an optimum level of the vitamin.

Using radioactive Co, Chow, Davis and Davis (1943) found that chlortetracycline increased the amount of vitamin B_{12} in the faeces by three-to eight-fold, but examination of the tissues for radioactivity indicated that very little vitamin B_{12} had been absorbed. This failure to utilize the increased vitamin B_{12} synthesized in the presence of antibiotics may be due to production of "pseudo"-vitamin B_{12}, which is microbiologically active, and so estimated, but which is inactive for animals. It is also possible that the vitamins synthesized at the lower part of the intestinal tract are not absorbed and, therefore, are not available to the animal. Similar results have been obtained with chicks (Monson, Dietrich and Elvehjem, 1952) where the addition of either succinyl–sulphathiazole, streptomycin, penicillin or bacitracin to a dextrin-containing diet did not affect the vitamin B_{12} content of the livers. There was also no effect on the pantothenic acid, pyridoxine or niacin contents of the liver, but there were two- to five-fold increases in folic acid and citrovorum factor.

The most consistent vitamin-sparing effect of antibiotics has been observed with thiamine in rats (Sauberlich, 1952; Lih and Bauman, 1951) and is greater with diets containing dextrin than sucrose. In these studies antibiotics gave no response when adequate quantities of the vitamins were present, but large growth increases were obtained on suboptimum thiamine levels. Penicillin proved to be the most effective antibiotic (Lih and Bauman, 1951; Guggenheim *et al.*, 1953; Sauberlich, 1952); streptomycin was less active, chlortetracycline and oxytetracycline were inactive, and chloromycetin appeared inhibitory (Lih and Bauman, 1951). Riboflavin requirements were less affected by antibiotics than those of thiamine.

As was pointed out earlier, the growth response to penicillin on a thiamine-free diet indicates that the antibiotic must increase intestinal synthesis. The response at intermediate levels of vitamins might be explained by a suppression of organisms which absorb vitamins and thus make them unavailable to the host, but such an explanation is not possible on thiamine-

is because there is interference in absorption or whether the worm itself either uses excessively or destroys the vitamin.

D. Bacterial Destruction of Vitamins

Intestinal bacteria may also destroy essential nutrients; for example, vitamin C is destroyed by *Escherichia coli in vitro* in the absence of more readily fermentable carbohydrate. It is known that certain scorbutic cases will respond to injected but not to oral vitamin C (Young, 1942; Young and James, 1942; Young and Rettger, 1943).

There is the evidence of Girdwood and Doig (1957) that an altered flora may occur in blind loops or sacs of the intestine, and in intestinal diverticula. Halstead, Lewis and Gasster (1956) have reported that the anaemia in the "blind loop" syndrome is unaffected by neomycin, which is not readily absorbed, whereas it may be cured by chlortetracycline, which is readily absorbed, and may therefore appear in sufficient concentration in the "blind loop".

Card (1959) points out that if there exists, under certain conditions in the intestine, an altered flora such that essential trace food factors are lost to the host, then the purely descriptive name of "blind loop" should be abandoned and replaced by some name which describes the functional state of privation. He asks if we dare regard the fatty liver, which proceeds to fibrosis, seen in some patients with severe ulcerative colitis as, in part, a nutritional disease of the liver due to involvement of the caecum and ascending colon, that is, that part of the gut which is said to be the main site of vitamin synthesis.

According to Jones (1959) this same mechanism operates in causing the steatorrhoea associated with gastrocolic fistula, and contributes to malabsorption after a Polya partial gastrectomy in multiple diverticulosis of the intestine, and in patients with Crotin's disease treated by short-circuiting procedures.

It is probable that the supplanting in man of bacteria which normally synthesize vitamins in the intestine, or the growth of bacteria which themselves utilize substances required by the host, might result in a deficiency state (Card, 1959).

The clinical problem of anaemia in relation to the small intestine depends on the interplay of impairment of absorption of iron, folic acid and vitamin B_{12}. Vitamin B_{12} is absorbed mainly from the lower end of the small intestine, and low levels arise in several ways. There may be a primary deficiency of intrinsic factor. There may be competition for B_{12} by intestinal bacteria or by *Dibothriocephalus latus*.

Folic acid, on the other hand, is absorbed mainly in the jejunum (Cox *et al.*, 1958), and iron in the first 18 in. of the small intestine.

Girdwood and Doig (1957) studied three patients with, respectively, small intestine fistulae, an ileo-transverse colostomy, and jejunal diverticulosis. Bacteria (mainly *E. coli*) were isolated from the stagnant areas at operation, and were shown to absorb vitamin B_{12} *in vitro* but to synthesize folic acid under such conditions. These patients had normal absorption of folic acid given by mouth.

The small intestine cannot, of course, absorb vitamin B_{12} unless the stomach secretes the intrinsic factor. If absorption in man is impaired and cannot be corrected by giving intrinsic factor, some disorder of small intestine function is likely. If the impaired absorption can be improved by giving antibiotics there is probably some anatomical lesion of the intestine permitting bacteria to flourish and utilize the vitamin. Absorption, according to Rowlands (1959), is often impaired in coeliac disease and regional ileitis, and it cannot be improved by giving intrinsic factor or antibiotics, but may be improved by steroid therapy.

Card (1959), summarizing the early work of Seyderhelm (1922), Tönnis and Brusis (1931, 1932), Pearce (1934), Cameron, Watson and Witts (1949) and others, stated that "the production of intestinal blind loops, sacs or strictures may profoundly affect an animal, but only when stasis has been produced; that these effects include malnutrition, anaemia, loss of weight, and may terminate in death; that the effects may be abolished by extirpation of the blind loop or sac, or the reaction of the stricture; and that improvement of the animal's condition may be achieved by intestinal antiseptics, more recently by antibiotics, and also by giving folic acid, liver injections, etc."

VII. ANTIBIOTICS

A. Effect of *Antibiotics on Nutritional Requirements*

The observation, by Stokstad *et al.* (1949), that the addition of aureomycin to the diet stimulated the growth of chicks, was of great interest. Similar observations were later made by numerous workers on pigs, chicks, and turkey poults using aureomycin terramycin or penicillin (Wahlstrom, Terrill and Johnson, 1950; Coates *et al.*, 1951; Sieburth *et al.*, 1951; Larson and Carpenter, 1952).

The inactivity of antibiotics for chicks in a germ-free environment (Luckey, 1952), and the failure of chlortetracycline to stimulate developing chick embryo

appearance of infection in newly-cleaned premises. When penicillin was fed to chicks, *Cl. welchii* was eliminated.

In an experiment with chicks from another source, the numbers of *Cl. welchii* were similar whether the chicks received penicillin or not, but the toxigenicity of the strains from the penicillin-treated group was markedly impaired after first isolation. In a further experiment the numbers and toxigenicity were reduced (Lev, Briggs and Coates, 1957).

1. Untoward Effect of Antibiotic in the Guinea-pig

In contrast, Roine *et al.* (1955) found that aureomycin was fatal to guinea-pigs when given in the diet at levels which normally promote growth in other species. The detrimental effect of aureomycin was accompanied by marked changes in the caecal flora, and, in particular, the numbers of *Listeria organisms* were considerably increased. *Listeria monocytogenes* is a known parasite of some domestic animals, and produces a syndrome similar to that observed in aureomycin-treated guinea-pigs.

C. Non-bacterial Mechanism of Action of Antibiotics

While the foregoing discussion has been based upon the assumption that antibiotics bring about their effect on growth through an action on microorganisms, there are some observations with which such an assumption is not compatible. Elam, Gee and Couch (1951) found that penicillin, made inactive for test-organisms by autoclaving, promoted growth when injected into chicks. Later, Fell and Stephenson (1953) obtained a growth response in chicks to penicillamine, which supports the suggestion, tentatively put forward by Elam *et al.*, that the antibiotic molecule, or a fragment of it, might act as a metabolite within the body of the bird. More recently, Taylor and Gordon (1955) showed that penicillin, inactivated by any of three different methods, retained a considerable proportion of its growth-promoting action for pigs. In these animals no penicillin could be detected in the gut, serum or urine. These authors suggest that penicillamine may be responsible for the growth effect being the only stable end-product. In contrast, Jowsey *et al.* (1957) found a lack of growth-promoting activity of inactivated penicillin (autoclaving or by penicillinase) with turkey poults.

Perhaps the most striking evidence for a non-bacterial mechanism of action is the work of Luckey *et al.* (1955), who obtained significant growth responses to antibiotics in germ-free chicks and poults. It has been suggested that in the earlier experiments of Luckey (1952), and Jukes and Williams (1953), where they failed to get growth stimulation, the absence of an effect may have been due to too high a dosage. But Forbes and Park (1959) found that a growth-promoting effect of dietary penicillin, in both conventional chicks and in chicks hatched and reared in the absence of bacteria and fungi, occurred when the animal room was deliberately infected with intestinal contents from chicks reared in premises where a growth response to antibiotics occurred regularly. Repeated experiments in clean premises had failed to reveal such a response in both germ-free and conventional animals.

It is presumed that the more recent finding indicates that the antibiotics produce their effect by direct action on the birds receiving them, rather than by virtue of their antibacterial properties. Coates and Kon (1955), in summing up, state: "It is generally accepted that antibiotics have a direct effect on certain metabolic processes, hence it is conceivable that, given in the diet, they exert a small but definite pharmacological action which contributes to their beneficial effect on growth, but that in ordinary environmental conditions such an action may pass unnoticed because of the overriding predominance of the bacteriological effect."

D. Emergence of Resistant Strains

Smith and Crabb (1957) found that although continuous treatment with dietary chlor- or oxytetracycline did not affect total counts of *Escherichia coli* in the faeces of pigs and chickens it caused a flora in which nearly all were of strains resistant to the drugs; nearly all in the faeces of untreated animals were sensitive to them. When sows were given tetracycline in their diets, and their milk was the only source of feed for their litters, the faeces of the piglets contained a high proportion of resistant strains. In pigs, the resistant strains developed rapidly after tetracycline was given, and persisted for as long as 7 months after the drug had been withdrawn.

A wide, random survey of pigs, poultry and sheep showed that in the gut of many pigs a large proportion of *E. coli* were resistant, whether the pigs had been systematically treated with antibiotics or not. This was probably because of the widespread practice of including tetracycline in feeds. In herds where this was done, diseases associated with *E. coli* were common, and the bacteria isolated from infected pigs were shown to be of resistant strains. Tetracycline did not affect the sensitvity of *E. coli* to other drugs.

It was concluded that, since permanent resistance could not be induced in non-resistant strains, the flora of predominantly resistant strains was due not to adaptation but to selection by elimination of sensitive strains. The practice of regularly using tetracycline in the ration was not considered wise, since the advan-

Whitehair (1952) has shown that baby pigs delivered aseptically by Caesarean Section and reared in isolation did not respond to aureomycin. In his summary of experiments on the use of antibiotics in pig feeding in the United Kingdom, Braude (1955) showed that where the general level of growth was poorest the greatest improvement resulted from antibiotics.

Lucas and Calder (1957) found that procaine penicillin improved rate of live-weight gain and feed efficiency of pigs by 3 per cent ($P<0{\cdot}10$) and 2 per cent (not significant) and housing (cold and bad piggery *v.* warm and good piggery) did not affect this response. Added vitamins (riboflavin, calcium pantothenate, and ascorbic acid) were of no benefit in warm pens, but improved rate of live-weight gain and feed efficiency in the cold pens by 5 per cent (P almost 0·05) and 4 per cent (P just 0·05) respectively. There was no evidence that antibiotic spared the need for extra vitamins in cold pens.

Experiments were carried out to investigate the effect on the rate of growth and food-conversion ratio of fattening pigs with various levels of penicillin and chlortetracycline in their diet. Rations were used in which antibiotic was added at the rate of 0, 2, 4, 8, 16, 32, 64, 128 and 256 g/ton food. The results suggest that there is improvement in these indices of effect with the dose level of antibiotic, at least with the range studied, and that the increase is substantially the same for penicillin and chlortetracycline. These results confirm those of Catron *et al.* (1951) on dosage effects, also the result of the trials sponsored by the Agricultural Research Council (1953). These experiments of Taylor and Rowell were carried out in animal accommodation which was very thoroughly cleaned before the beginning of the experiment, and the buildings had housed pigs for only a short time before the experiment. Under practical farming conditions, with the usual degree of environmental contamination, it has been found that the broad spectrum antibiotics may be of most value. Thus, Braude, Wallace and Cunha (1953) suggested that, under normal conditions, chlortetracycline may be some 30 per cent more effective than penicillin in increasing the rate of growth of pigs.

In opposition to the widely-held idea that antibiotics may eradicate organisms detrimental to growth, the possibility exists that antibiotics may encourage the development of an internal microflora of benefit to animal growth. Once again, different environments may favour the establishment of different organisms in the gut, hence, directly or indirectly, influence the nutrition of the host. The report by Romoser *et al.* (1952) of the appearance of *Aerobacter aerogenes* in the caeca of chicks given penicillin was followed by further experiments (Romoser, Shorb and Combs, 1952) in which the feeding of a viable culture of this organism to chicks resulted in a growth response that partly replaced the effect of antibiotics. In somewhat parallel experiments Anderson, Slinger and Pepper (1953) gave live cultures of certain strains of *Escherichia coli* to chicks and to poults (Anderson *et al.*, 1953) and obtained weight increases in the absence of penicillin. No organisms of the genus *Aerobacter* were encountered in the caeca of their chickens. Thus improved growth was associated with distinct bacterial "milieux." Antibiotics in the diet may assist the establishment of the critical flora but, in environments where the appropriate organisms already flourish, their use would appear to be redundant.

As we have seen, there is strong reason to believe that the effect of an antibiotic is mediated through the gut flora as Jukes has indicated (Jukes, 1955). Varied and sometimes contradictory results have been obtained by different groups of workers. For example, Dixon and Thayer (1951) reported an increase in the numbers of lactobacilli when they gave chlortetracycline to chicks. March and Biely (1952) reported a decrease, whereas Eisenstark and Sanford (1953) and Anderson, Cunningham and Slinger (1953) found no change.

In considering the nature of the growth-depressing condition in "stall" premises, Coates and Kon (1955) point out that a change in bacterial metabolism rather in types or numbers may be responsible. Such a phenomenon would not be easily disclosed by ordinary bacteriological techniques, but its existence might explain the striking lack of agreement between the results of different groups of workers who have tried to identify a specific organism concerned in growth stimulation by antibiotics. For instance, Smyser *et al.* (1952) in contrast to Elam *et al.* (1954) reached the conclusion that *Clostridium perfringens* has no significant role in stimulation of chick growth by dietary antibiotics. It is possible that, even if sub-maximal growth in the absence of antibiotics is the result of a specific agent, this may not be the same in all environments.

In a study of the anaerobic flora of the caeca of "infected" and "uninfected" and penicillin-fed birds during the first few days of life, since an "adult" flora is established in chicks 2 days after they begin to feed (Lev and Briggs, 1956*b*), no differences were found in total numbers of lactobacilli, streptococci or coliforms in infected and uninfected groups of chicks when six sites in the gut were sampled. Distinct differences were found in the clostridial population between infected and uninfected groups. *Clostridium welchii* appeared 1 day after feeding in the caeca of the infected but not of the uninfected groups. The presence of *Cl. welchii* was used as an index of the re-

F. Effects of Antibiotics on Host Tissues

Coates and Kon (1955) have reviewed the evidence that antibiotics lead to a decrease in weight of the gut and find ample confirmation for this finding. Coates, Davies and Kon (1955) showed that thinning, rather than shortening, is the cause, and consider that the thicker gut is a reaction to the establishment of an unfavourable flora. It might conceivably result in less efficient absorption. Jukes, Hill and Branion (1956) have observed that the average weight of the intestinal tract of chickens given antibiotics in their ration was less than that of untreated birds: the tunica propia was thinner. When penicillin, from 2 to 100 mg per lb was added to a casein and cerolose, or maize and soya bean diet, the weight of the intestine was decreased by amounts of antibiotic too small to affect body weight (Hill, Keeling and Kelly, 1957).

After partial hepatectomy, estimated liver regeneration was less in rats and growing chicks which had received aureomycin in their diets than in those which had not; the aureomycin-treated chicks were heavier than the untreated. It is suggested by Calet (1958) that the depressed protein metabolism previously found in aureomycin-treated animals is due to reduction of activity and cell multiplication in the liver.

VIII. GERM-FREE LIFE

Some mention of this interesting aspect of microbiology has already been made. The early history of germ-free life methodology (gnotobiotics) and experimental nutrition have been reviewed by Reyniers (1955), and his own technique thoroughly explained. According to Reyniers (1955) the basic physiological functions of the intestinal canal in the germ-free animal, together with digestion, are not, so far as we know, different from the conventional animal. Reyniers *et al.* (1950) have shown that, at least in the germ-free chick, no new nutritional factors are needed but that deficiencies can be obtained by withdrawal of individual B-vitamins (Luckey, Pleasants and Reyniers, 1955). It can also recover spontaneously from vitamin-K deficiency. It may be assumed that the need for these vitamins is no different from that of the conventional animal. Also, since the excreta of deficient germ-free birds contain appreciable quantities of the vitamins in question which, if given orally or injected, would save their lives, and since by definition these vitamins cannot be due to synthesis by micro-organisms, it is probable that, in the normal animal, intestinal synthesis by them in the large gut is relatively less important to the host than was originally thought. These studies were made with first-generation animals, *i.e.* not bred from germ-free parents. A study of sixth-generation germ-free rats (Luckey *et al.*, 1955) indicated, with an occasional exception, that the germ-free metabolism is very similar to its conventional counterpart.

In germ-free rats a source of biotin is required, and less niacin, riboflavin and biotin were excreted and accumulated in body tissues than were fed. On the other hand, quantities of pantothenic acid and vitamin B_{12} excreted and accumulated were equal to the amounts fed. Biosynthesis of inositol was indicated, and vitamin B_{10} (measured as folic acid and citrovorum factor) was synthesized. But these studies are by no means complete.

Reyniers (1955) has suggested that in the present state of knowledge the following effects need more detailed study:

(i) At birth, and through life, micro-organisms may act by (*a*) altering intestinal permeability, (*b*) affecting the tonus of the tract, (*c*) changing the motility, (*d*) affecting the structure of the intestinal wall, and (*e*) affecting the accessory digestive organs such as the liver.

(ii) Micro-organisms may change the physical–chemical environment of the digestive tract, *i.e.* pH, oxygen tension, fluid contents, osmotic balance, etc.

(iii) Micro-organisms may alter the supply of blood and blood elements, *e.g.* lymphocytes, to the digestive tract.

(iv) The nutrition of the host may be influenced by (*a*) incorporating essential nutritional elements in the microbial protoplasm, (*b*) unbalancing available and needed supplies of nutrients or metabolites, (*c*) providing nutritional elements through possible intestinal synthesis, and (*d*) aiding in breaking down foodstuffs such as cellulose.

(v) Production of toxic substances by bacteria which, if absorbed through the intestinal wall, may affect the health of the animal and, therefore, its nutrition.

(vi) Bacteria may condition the animal by activating body defences and enhancing or specializing these defences in emergencies with a reflection in the intestinal wall and possibly in the endocrine system.

(vii) The conventional intestinal tract may provide a reservoir of infective agents either in the intestinal canal, or in the tissues and other systems which will threaten the health of the host.

(viii) If the defence mechanism and reaction systems are not stressed by the action of micro-organisms, there may be less burden on the animal with respect to its nutritive requirements.

tages of growth stimulation might be offset by complications caused by the widespread establishment of predominantly resistant flora.

Barnes (1958) also found that resistant strains of faecal streptococci developed in poultry fed rations containing chlortetracycline.

E. Primary Action of Antibiotics against Micro-organisms

As Topley and Wilson (1955) have pointed out, many attempts have been made to answer the question "Does an antibiotic induce resistance in a growing culture of sensitive bacteria by selecting naturally-resistant mutants, or by impressing on the cells an adaptation that is heritable?"

The mechanisms whereby the antibiotics effect their cytotoxic action remains obscure. Whether with penicillin, streptomycin, or the so-called broad-spectrum antibiotics, the large number of observations describing effects on metabolic activities cannot yet be incorporated into a coherent and meaningful pattern. The difficulty of distinguishing between a biochemical change which is a direct effect of the antibiotic, and a secondary effect in a damaged cell continues to complicate the interpretation of the data.

The enhancement of penicillin activity by cobalt (Pratt and Dufrenoy, 1948) has been found to apply to streptomycin, bacitracin and penicillin–streptomycin combinations but not to chloramphenicol, chlortetracycline, or polymyxin (Trace and Edds, 1954; Trace, 1955). Similarly, cobalt has been found to potentiate the action of tyrothricin and bacitracin in combination (Forni and Ruggenini, 1954). There seems to be increased vulnerability of some bacteria to the host after their exposure to the antibiotics. Radisson *et al.* (1956) found that bacteria isolated from the faeces of calves receiving aureomycin were more sensitive to phagocytosis than bacteria isolated from the faeces of calves receiving no aureomycin. It is possible that part of the effect of antibiotics on growth is due to their effect on the physiology of the bacteria rather than on the numbers of intestinal bacteria.

According to Eagle and Saz (1955) there is overwhelming evidence that the highly resistant organisms which grow out selectively in the presence of high concentrations of an antibiotic, and which retain their resistance after cultivation for many generations in drug-free media, are in fact initially present in the culture and do not arise as a result of their exposure to the drug (Demerec, 1945, 1948, 1949; Newcombe, 1949; Lederberg, 1951; Newcombe and Nyholm, 1950; Lederberg and Lederberg, 1952; Hotchkiss, 1951; Alexander and Leidy, 1953; Zinder and Lederberg, 1952). In the aggregate, the lines of evidence quoted, and others not here cited by these two workers, indicate, apparently incontrovertibly, that resistant variants are in fact preformed mutants which grow out selectively in the presence of the antibiotic.

No explanation has been offered for the decreased susceptibility of resistant variants to normally effective concentrations of antibiotics. In no case is the development of penicillin resistance associated with an increased capacity to inactivate the antibiotic (Eagle, 1954).

Although it seems clear that antibiotic-resistant staphylococci have greatly increased in prevalence as a result of antibiotic therapy, it seems equally clear that in many instances, and perhaps in most, the increased resistance does not represent an adaptive change or a mutational selection within strains which were originally sensitive. Indeed, there appears to have been a selective propagation of strains which were resistant to start with and which have grown out selectively in a host environment which limited the growth of the normally preponderant sensitive strains.

It is suggested that the medical and nursing staffs of hospitals and similar centres of antibiotic therapy have served as carriers of these resistant strains.

The enhancement of the virulence of *Candida albicans* by chlortetracycline observed by Seligmann (1952) has now been observed with all three tetracyclines (Dukes and Tettenbaum, 1955).

Park and Strominger (1957) and Park (1958) have shown by direct demonstration that bacterial lysis, "protoplast" formation, and accumulation of cell wall precursors occur in the presence of penicillin, indicating that the primary target of its action is the cell wall structural unit. Selective inhibition of cell wall synthesis has also been observed with compounds such as oxamycin, bacitracin or glycine which, like penicillin, are known to cause "protoplast" formation or accumulation of wall precursors.

Specific inhibition of bacterial protein synthesis, not only by chloramphenicol but also by chlortetracycline and oxytetracycline, has been demonstrated by Gale and Folkes (1953). However, other biosynthetic processes, notably the synthesis of both types of nucleic acid continues (Wisseman *et al.*, 1954). For many strains these events result in a clear-cut bacteriostasis. It is not implied that the actual mechanisms of action on the molecular level are the same for chloramphenicol and for tetracyclines.

As it is currently thought that the initial step in protein synthesis is the formation of aminoacyl adenylates from amino acids and ATP which is followed by a transfer of the activated amino acids to RNA (Lipmann, 1958), this activation of the amino acids and their transfer to RNA might conceivably be steps in protein synthesis that are susceptible to chloramphenicol (Hahn, 1958).

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5

The Vitamin Interrelations of Ascorbic Acid

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Contents

INTRODUCTION

The vitamin interrelations of ascorbic acid are particularly striking, both on account of the large number of vitamins with which this factor is in liaison, and of the nature of this liaison.

Ascorbic acid has a connexion with many vitamins in group B, also with vitamins E and A. The existence of this connexion is shown particularly by the facility with which ascorbic acid is able to replace, more or less efficiently, all the vitamins with which it is in relation when one or other is lacking in the diet. In this respect, it is the important work of Dam and his school, and that of Daft and his collaborators, which first drew attention to the remarkable protective role of ascorbic acid in certain vitamin deficiencies.

The facts showing the more or less active exchangeability of ascorbic acid with the numerous vitamins are set out in Part 1. Only the results of experiments on animals or micro-organisms are given. The clinical observations on humans, whose interpretation is obviously much more complex, have been left aside. Part 2 is concerned with bringing to light mechanisms and characteristics of this substitutional power of ascorbic acid in various vitamin deficiencies.

PART ONE: FACTS SHOWING THE PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN DEFICIENCIES

The most obvious demonstration of the protective power of ascorbic acid against vitamin deficiency, and the only one dealt with here, apart from rare exceptions, depends always on the same experimental principles: (1) The supplementing of a diet totally deprived of a given vitamin by ascorbic acid. According to whether its preventive or curative protective power against vitamin deficiency is to be shown, ascorbic acid is administered either at the outset of the deficiency, and during it, or only when the deficiency is well established. (2) Observation of the consequences of this supplementing on all aspects of the deficiency. These can be subdivided into two groups: (i) the manifestations, common to all deficiency, which affect growth and the survival time; (ii) the apparent "clinical" symptoms and the metabolic symptoms, both more or less specific to a particular deficiency.

Little systematic work of this kind has been undertaken as yet, that is, the problem of the substitution of ascorbic acid for vitamins is an open field for much indispensable additional investigations.

Work of very unequal value nevertheless makes it possible to establish the number of vitamins of group B which, in some connexion, can be replaced by ascorbic acid, at six. These are thiamine, riboflavin, pantothenic acid, biotin, folic acid and vitamin B_{12}.

The substitution of ascorbic acid for the other factors of group B—nicotinic acid, pyridoxine, choline, para-aminobenzoic acid, etc.—remains to be investigated or has been only little studied.

On the other hand, ascorbic acid exercises a protective activity incontestable on E deficiency, but debatable on A deficiency.

Without claiming to be complete, the following account groups together certain facts relating to the substitutional power of ascorbic acid in the vitamin deficiencies listed above.

I. PROTECTIVE POWER OF ASCORBIC ACID AGAINST THIAMINE DEFICIENCY

A. Preventive Protective Power of Ascorbic Acid against Thiamine Deficiency

The existence of a protective activity of ascorbic acid against thiamine deficiency is brought out clearly by the two following preliminary experiments, both carried out on the rat. First, young rats receiving only one-sixth of the necessary thiamine nevertheless maintain absolutely normal growth with a daily administration of 10 mg ascorbic acid per animal (Kasahara *et al.*, 1939). Later, Daft and Schwarz (1952) mention, in one phrase in a short note, that the "introduction of 3 per cent ascorbic acid in the diet of B_1-deficient young rats made it possible to delay or attenuate the signs of the deficiency."

A detailed analysis of this remarkable protective power shows, as will be seen, that it extends to all the aspects of B_1 deficiency, apparent, metabolic and endocrinian symptoms.

1. Preventive Protective Power of Ascorbic Acid against the Apparent Symptoms of Thiamine Deficiency

(a) *Growth*

The body weight of young rats with vitamin-B_1

decrease of appetite. Ingestion which is approximately 8 g dry/day/rat in the two first weeks of deficiency, then falls to 4 g dry. Now, at the same time, ingestion of animals with thiamine deficiency, but receiving 3 per cent ascorbic acid, is maintained at 9 g dry/day/rat. The quantities ingested, thanks to the ascorbic acid, remain, however, inferior to normal, which is 10 to 12 g dry in the control rats with *ad lib.* diet. Later, they drop more, in a line parallel to the later body weight decline. Nevertheless, although very considerable, the stimulation, by ascorbic acid, of the appetite of young animals deprived of thiamine does not seem, at first sight, to be able to explain, at any rate by this alone, the protective role of vitamin C. Indeed the pair-fed control rats receiving a qualitatively complete, but quantitatively restricted ration to that ingested by the rats deficient in thiamine and not supplemented with ascorbic acid, do not present, except for the body weight fall, any of the characteristic symptoms of vitamin-B_1 deficiency (Terroine, T., 1957*a*).

(c) *Nervous Disorders*

Nervous symptoms, stumbling walk, and violent convulsions, come a few days before death. Ascorbic acid in very strong or moderate doses does not eliminate them, but delays their appearance or attenuates their gravity in the rat (Bhattacharya, 1957; Terroine, T., 1957*a*).

(d) *Survival Time*

Half the rats from the age of about one month *submitted to a total thiamine deficiency die by the end of four weeks' treatment, and the extreme date* of survival time does not exceed eight weeks. But, when 3 per cent ascorbic acid is added to the diet deprived of thiamine, death does not appear before the twelfth week; it may be complete at the end of the fourteenth week, but it may also exceed considerably this limit (Terroine, T., 1957*a*). Scott and Griffith (1957) also observed that their rats with thiamine deficiency which were receiving 5 per cent ascorbic acid, were all living at the twelfth week of the experiment. Even with a moderate dose of 500 μg ascorbic acid/day/animal the survival time of young rats with vitamin-B_1 deficiency is prolonged slightly by a week (Bhattacharya, 1957).

On the whole, it also appears that the average body weight at the time of death is definitely superior in deficient animals receiving ascorbic acid. It is 112 g against 72 g in the group submitted to ordinary B_1 deficiency (Terroine, T., 1957*a*).

2. Preventive Protective Power of Ascorbic Acid against the Metabolic Symptoms of Thiamine Deficiency

(a) *Hyperpyruvicemia*

Ascorbic acid, administered at a rate of 3 per cent in the thiamine-deficient diet, strongly inhibits hyperpyruvicemia in the rat, a disorder which, if not specific, is at least characteristic of vitamin-B_1 deficiency. This is shown by Table 5.1, which refers to young rats *after 35 days' treatment (Terroine, T., 1957a).*

TABLE 5.1*

Consequences of the Administration of Ascorbic Acid on Hyperpyruvicemia, Hypercreatinuria and Hypercreatininuria of Rats Deficient in Thiamine*

Nature of diets	Weight of rats	Ingested dry weight g/day	Blood pyruvic acid mg p. 100 ml	Urinary creatin mg/100 g/24 hr	Urinary creatinin mg/100 g/24 hr
Control diet given *ad lib.*	193	12	—	0·77 (0·57–1·09)	4·97 (4·65–5·56)
Control diet given *in limited quantity*	110	3·5	1·70 (0·75–2·22)	1·75 (0·89–3·34)	4·70 (3·54–5·6)
Diet deficient in thiamine	83	3·5	8·70 (7·2–11·8)	7·21 (4·02–9·76)	8·38 (7·08–10 0)
Diet deficient in thiamine +3 per cent ascorbic acid	160	9·5	3·51 (3·0–5 0)	1·61 (1·19–2·03)	6·63 (5·33–8·84)

* According to Terroine, T., (1957*a*).

deficiency is remarkably stimulated, as shown by Fig. 5.1, by the addition to the deficient diet of 3 to 5 per cent ascorbic acid (Scott and Griffith, 1957; Terroine, T., 1957*a*).

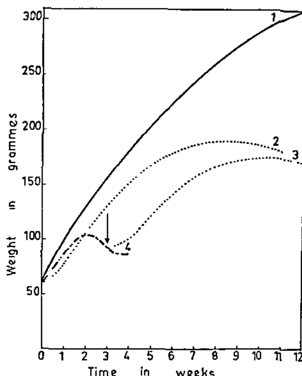


FIG. 5.1. PREVENTIVE AND CURATIVE INFLUENCES OF ASCORBIC ACID ON THE BODY WEIGHT OF RATS DEFICIENT IN THIAMINE

1 = normal diet given *ad lib.*, 2 = thiamine-deficient diet supplemented with 3 per cent ascorbic acid throughout the deficiency; 3 = thiamine-deficient diet supplemented with 3 per cent ascorbic acid only, after three weeks, as is shown by the arrow; 4 = thiamine-deficient diet without addition of ascorbic acid (according to *Terroine, T.*, 1957*a*).

This favourable action of ascorbic acid does not, however, seem to be perfect; it is very unequal from one animal to another; it does not re-establish normal growth (growth always remains on an average very inferior to that of animals control fed *ad lib.*); and, what is not mentioned by Scott and Griffith (1957), it is not lasting. At the end of a variable period, nine weeks in Fig. 5.1, the body weight of animals deprived of thiamine and receiving ascorbic acid, not only ceases to increase, but even begins to decline (Terroine, T., 1957*a*).

In spite of the above remarks, the ability of ascorbic acid to improve the body weight of rats with vitamin B_1 deficiency is nevertheless spectacular. This ability of ascorbic acid extends also to deficient adult rats (Terroine, T., 1957*a*), and begins to be apparent with doses 600 times weaker than those referred to above, as was observed by Bhattacharya (1957) in young rats with vitamin-B_1 deficiency.

Finally, the stimulating action of ascorbic acid on growth takes place in a micro-organism, the *Lactobacillus fermenti* 36, placed in a medium totally deprived of, or only low in thiamine (Fang and Butts, 1953; Terroine, T., 1957*a*). However, as Fig. 5.2 shows, the addition of a strong dose of ascorbic acid to a low thiamine medium did not succeed in bringing the growth of the micro-organism up to its normal level (Terroine, T., 1957*a*, 1959*a*).

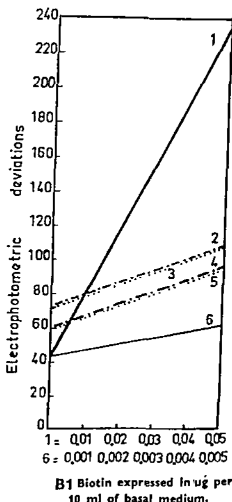


FIG. 5.2. INFLUENCE OF ASCORBIC ACID AND OF ISOASCORBIC ACID ON THE GROWTH OF *Lactobacillus Fermenti* 36 IN A MEDIUM LOW IN THIAMINE

Growth of *L. Fermenti* 36 with: 1 = the optimum concentrations of thiamine; 6 = the 1/10 of the optimum concentrations. Growth with the previous sub-optimum concentrations supplemented per ml of basal medium with: 4 = 2 mg isoascorbic acid; 5 = 2 mg ascorbic acid; 2 = 5 mg isoascorbic acid; 3 = 5 mg ascorbic acid (according to *Terroine, T.*, 1957*a*, *Terroine, T.*, 1959*a*).

(b) *Appetite*

More than the other vitamin deficiencies, deficiency of B_1 is accompanied by a rapid and considerable

considerable improvement of the body weight (Fig. 5.1) and a simultaneous increase in appetite. But the curative power of ascorbic acid, as well as its preventive power, is incomplete and transitory (Terroine, T., 1957*a*).

Thus, on the whole, on the basis of the experimental results, which could be extended to more numerous species of animals, it seems possible to replace thiamine with great effectiveness, at least temporarily, by ascorbic acid.

II. PROTECTIVE POWER OF ASCORBIC ACID AGAINST RIBOFLAVIN DEFICIENCY

Preventive Protective Power of Ascorbic Acid against Riboflavin Deficiency

1. Preventive Protective Power of Ascorbic Acid against the Apparent Symptoms of Riboflavin Deficiency

(a) *Growth*

The addition of 2 per cent ascorbic acid to the riboflavin-deficient diet improves very considerably the body weight of young rats (Daft and Schwarz, 1952; Terroine, T., 1957*b*). The growth is superior, as is shown by Fig. 5.3, by 20 to 30 per cent to the deficient non-supplemented group; but it remains, on the other hand, inferior by 25 to 40 per cent to its value in normal animals. This improvement of the body weight of the group deficient in riboflavin, but receiving ascorbic acid, still persists after two months' treatment (Fig. 5.3; Terroine, T., 1957*b*).

The aptitude for ascorbic acid to stimulate growth seems, however, less positive in riboflavin deficiency than in thiamine deficiency. Moderate doses of ascorbic acid—about 1,000 μg/day—were not able here, as they were able in vitamin-B_1 deficiency, to improve the body weight of rats deprived of riboflavin (Ekman and Strombeck, 1949). On the other hand, the only assay carried out on micro-organisms also proved negative. The very limited development of *Lactobacillus casei*, in a culture medium containing only 1/10 or 1/2 the optimum concentration of riboflavin was in no way stimulated by the addition of ascorbic acid at a rate of 500, 2,000 or 5,000 μg/10 ml of this medium (Terroine, T., 1957*b*).

(b) *Appetite*

Although in a less spectacular manner than in thiamine deficiency, ascorbic acid, added at the rate of the 2 per cent to the diet, considerably increases the appetite of young rats deprived of riboflavin. After two months' treatment, ingestion increases to 11·7 dry g/day/animal in the latter group, while it is only 7·8 in the subjects deprived of both B_1 and ascorbic acid. At the same time, ingestion of rats control fed *ad lib.* is 13·6 g. The coefficient of use of the ration, that is, the relation of gain in body weight to the quantities ingested is, respectively, in the three categories of groups: 0·18, 0·14 and 0·23 (Terroine, T., 1957*b*).

(c) *Survival Time*

Daft and Schwarz (1952) kept alive, for a year, rats supplemented with ascorbic acid. With a moderate dose of ascorbic acid—1 mg/day/rat—the survival time of animals is slightly prolonged, by a week (Ekman and Strombeck, 1949).

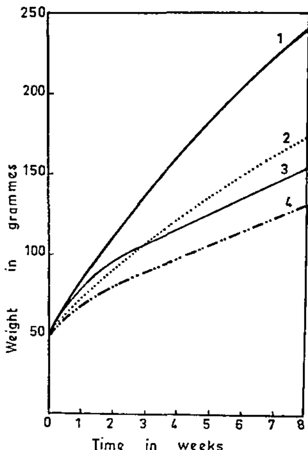


Fig. 5.3. Preventive Influence of Ascorbic Acid on the Body Weight of Rats Deficient in Riboflavin

1 = normal diet given *ad lib.*; 2 = riboflavin-deficient diet supplemented with 2 per cent ascorbic acid throughout the deficiency; 3 = normal diet given in restricted quantity equal to that ingested by the deficient group without ascorbic acid; 4 = riboflavin-deficient diet without addition of ascorbic acid (according to *Terroine, T.*, 1957*a*).

The protection brought by ascorbic acid against hyperpyruvicemia is nevertheless incomplete, since the concentration of blood pyruvic acid in the thiamine-deficient group receiving ascorbic acid still remains double the normal amount. With a much weaker dose of ascorbic acid—500 μg/day/rat—Bhattacharya (1957) no longer observes, on the other hand, any attenuation in the hyperpyruvicemia of rats with vitamin-B_1 deficiency.

(b) *Urinary Hyperexcretion of Creatinic Substances*

As for hyperpyruvicemia, hypercreatinuria, without being a specific symptom of thiamine deficiency, is nevertheless a characteristic disorder. This hypercreatinuria appears to be completely eliminated, since, as the figures of Table 1 show, creatinuria of the group with vitamin-B_1 deficiency, when supplemented with 3 per cent ascorbic acid, is equal to that of the pair-fed group. However, it should be noted that the comparison between these two groups leads to a certain confusion, since the alimentary restriction causes a certain creatinuria in the pair-fed control group.

In fact, from comparisons made, not with young rats but with adults in which creatinuria is 0·60 mg/100 g/24 hr, it is seen that the creatinuria of thiamine-deficient subjects receiving ascorbic acid is not entirely brought up to normal. It can be said, finally, that hypercreatinuria observed in rats deprived of thiamine is very characteristic of this deficiency. Indeed, the determinations were always made at least six days before death, and often much before. The animals showed, at the time of estimation, no nervous symptoms characteristic of the last phase of thiamine deficiency. The hypercreatinuria observed can therefore not be imputed to nitrogen metabolism disorders appearing in the immediate phase before death.

Only very slightly does ascorbic acid tend to reduce the hypercreatinuria of vitamin-B_1 deficiency (Terroine, T., 1957*a*).

3. Preventive Protective Power of Ascorbic Acid against the Endocrinian Symptoms of Thiamine Deficiency

The observations have been made on the thyroid gland, the adrenals and the genital tract of young rats submitted, for about 35 days, to a vitamin-B_1 deficient diet, without and with 3 per cent ascorbic acid.

(a) *Thyroid Gland*

The rats with vitamin-B_1 deficiency receiving ascorbic acid show, at a histological examination, a thyroid gland whose activity is only slightly inferior to that of a control group fed *ad lib.* The thyroid gland of animals deprived of both thiamine and ascorbic acid shows, on the other hand, a clearly inferior behaviour, which has already been observed by Carpenter and Sharpless (1937), Hundhausen and Schulze (1938) and Blaizot (1949). However, this inferior behaviour does not seem to be specific for thiamine deficiency, for it is seen to be comparable to that shown by the control animals whose alimentation is quantitatively limited to that ingested by the animals with vitamin-B_1 deficiency and without ascorbic acid. The protective action of ascorbic acid against the inferior behaviour of thyroid gland in vitamin-B_1 deficiency seems to be due, at least to some extent, to the maintenance of a subnormal appetite, the importance of which has already been indicated (Delost and Terroine, T., 1958*a*, 1959).

(b) *Adrenals*

Vitamin-B_1 deficiency modifies the structure of the zones of the adrenal cortex: decrease of the fasciculata zona, hypertrophy of the reticularis zona, atrophy of the glomerulosa zona. Only the last-named modification appears to be specific for thiamine deficiency, for it does not occur in the restricted control group. Now, all these changes of adrenal cortex are, to a large extent, prevented by ascorbic acid (Delost and Terroine, T., 1958*b*, 1959).

(c) *Male Genital Tract*

The ponderal development of the testis, the appearance of spermatogenesis, and the secretion of the interstitial gland are considerably delayed by thiamine deficiency. This inhibition of the male genital tract is not entirely due to malnutrition, according to Delost and Terroine, T. (1958*c*, 1959), for it is much more accentuated than in pair-fed controls. Here again, ascorbic acid noticeably improves the behaviour of the male genital tract in rats deprived of thiamine (Delost and Terroine, T., 1958*c*, 1959).

B. Curative Protective Power of Ascorbic Acid against Thiamine Deficiency

The curative action of ascorbic acid against thiamine deficiency has been observed by Kasahara *et al.* (1939) on the pigeon. These authors show that the association of only 1/20 of the necessary curative dose of thiamine with 60 mg ascorbic acid cures, in three hours, the characteristic nervous disorders of the pigeon, and quite as effectively as the optimum dose of thiamine. However striking this may be, this experiment nevertheless presents a certain ambiguity, since the curative power of ascorbic acid has not been tried alone, but only in combination with that of thiamine, however small a dose of the latter used.

Administered in heavy doses, to rats submitted this time to a strict and definite diet of vitamin-B_1 deficiency, ascorbic acid produces an immediate and

III. PROTECTIVE POWER OF ASCORBIC ACID AGAINST PANTOTHENIC ACID DEFICIENCY

A. Preventive Protective Power of Ascorbic Acid against Pantothenic-acid Deficiency

1. Preventive Protective Power of Ascorbic Acid against the Apparent Symptoms of Pantothenic-acid Deficiency

(a) *Growth*

Ascorbic acid, added at the rate of 2 to 4 per cent to the deficient diet, stimulates very strongly the growth of young rats deficient in pantothenic acid (Daft, 1951; Daft and Schwarz, 1952; Hundley and Ing, 1953; Barboriack and Krehl, 1957). However, as in the previous deficiencies, and as seen in Fig. 5.4, ascorbic acid does not succeed in raising the growth to the normal level observed in the controls fed *ad lib.* (Terroine, T., 1959*b*).

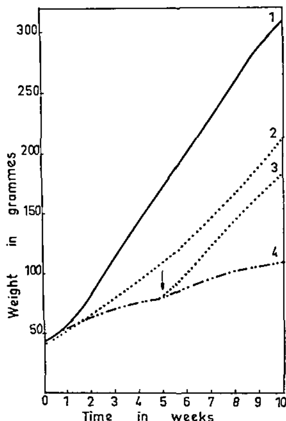


Fig. 5.4. Preventive and Curative Influences of Ascorbic Acid on the Body Weight of Rats Deficient in Pantothenic Acid

1 = normal diet given *ad lib.*; 2 = diet deficient in pantothenic acid supplemented with 3 per cent ascorbic acid throughout the deficiency; 3 = diet deficient in pantothenic acid and supplemented with 3 per cent ascorbic acid only, after five weeks, as indicated by the arrow; 4 = diet deficient in pantothenic acid without addition of ascorbic acid (according to *Terroine, T.*, 1959*b*).

Ascorbic acid also extends its aptitude for stimulating growth in pantothenic-acid deficiency to certain micro-organisms. Added at the rate of 2 mg/10 ml of medium containing only one-half the optima concentrations in pantothenic acid, it increases considerably the growth of *L. arabinosus* 17/5, though it does not bring it systematically to a normal rate. It is, however, unable to promote the growth of *L. casei* placed in the previous conditions (Terroine, T., 1958, 1959*a*).

(b) *Appetite*

As in the previous deficiencies, ascorbic acid improved the appetite of rats deprived of pantothenic acid. It ensured them of an ingestion of 10·9 dry g/day as against only 7 g without it. The *ad lib.* fed controls ingested, in the same time, 15 dry g/day (Terroine, T., 1959*b*).

(c) *Cutaneous Disorders*

The apparent clinical symptoms which are most characteristic of pantothenic-acid deficiency are: in the albino rat, a generalized cutaneous excretion of porphyrine, giving to the whole pelt and to the whiskers, a characteristic rust colour; in the black or piebald rat, a depigmentation of the fur, which turns from black to grey, occurs. This is the phenomenon of acromotrichy. The protection against this seems to be uncertain, according to Hundley and Ing (1953), and incomplete after 45 days' treatment, according to Barboriack and Krehl (1957). Ascorbic acid attenuates considerably without entirely preventing the porphyrine cutaneous excretion (Daft, 1951; Terroine, T., 1959*b*).

(d) *Survival Time*

Ascorbic acid undeniably prolongs the survival time of rats deprived of pantothenic acid. Indeed, the deficient animals treated in this way were all living after six or ten weeks of vitamin deficiency (Hundley and Ing, 1953; Terroine, T., 1959*b*). Most of them were also alive at the end of one year, some rats attaining a weight of 500 g (Daft and Schwarz, 1952).

2. Preventive Protective Power of Ascorbic Acid against the Metabolic Symptoms of Pantothenic-acid Deficiency

Hyperpyruvicemia

Functionally, as the work of Lipman has chiefly shown, pantothenic acid participates in the general oxidation process of the ternary chains by acetylizing

2. Preventive Protective Power of Ascorbic Acid against the Metabolic Symptoms of Riboflavin Deficiency

(a) *Disorders of the Metabolism of the Trytophan*

One of the specific metabolic symptoms of riboflavin deficiency is concerned with the disorder of the tryptophan metabolism. This disorder is characterised by the following modifications: important decrease of the conversion of the tryptophan and kinurenine into nicotinic derivatives (Henderson *et al.*, 1951); increase of urinary excretion of xanthurenic acid, on the one hand, and anthranilic acid and two other derivatives, on the other hand (Charconnet-Harding *et al.*, 1952; Mason, 1953).

Now, addition to the diet of 2 per cent ascorbic acid throughout the riboflavin deficiency totally inhibits the anomalies of the metabolism of the tryptophan produced by this deficiency (Charconnet-Harding and Terroine, T., 1956). As shown by Table 5.2, the animals submitted to a diet deprived of riboflavin, but with ascorbic acid added, respond normally to a large addition of DL tryptophan, leaving in the urine no abnormal accumulation of anthranilic and xanthurenic acids.

The favourable influence of ascorbic acid grows progressively stronger; after two months' treatment the excretion of xanthurenic acid is nil, while after one month it was slightly positive.

In conclusion, the general state of the rats deprived of riboflavin is decidedly improved by the administration of strong doses of ascorbic acid. Thus, a number of subjects treated in this way presented no cutaneous lesion after two months of the diet, while the animals not receiving ascorbic acid already showed very considerable alopecia.

However, as in thiamine deficiency, a considerable percentage of riboflavin-deficient rats miss the beneficial action of ascorbic acid, though the cause is not known (Daft and Schwarz, 1952).

Although undeniable, the protective role of ascorbic acid against riboflavin deficiency should be the object of further research, notably in seeking to show this, by using different species of animals, from both the preventive and curative angle.

TABLE 5.2*

Influence of Ascorbic Acid on the Reaction of Rats Deficient in Riboflavin to the Administration of a Single Dose of 150 mg of dl-tryptophane

Nature of diets	No. of rats	Weight of rats		Urinary xanthurenic acid†		Urinary anthranilic acid‡ after 60 days of treatment		
		Treatment		Treatment				
		after 30 days	after 60 days	after 30 days	after 60 days	Free anthranilic acid	In form of aminohippuric acid	In form of glucuronate
Control diet given *in limited quantity*	1	111	126	−	−	−	−	−
	2	125	126	−	−	−	−	−
	3	115	120	−	−	−	−	−
Diet deficient in B_2	4	97	86	+++	++++	+	+	+
	5	80	85	+++	++++	−	−	+
	6	87	122	+++	++++	+	+	+
	7	123	112	++	++++	+	+	+
Diet deficient in B_2 and supplemented with ascorbic acid in excess	8	160	198	+	−	−	−	−
	9	135	188	−	−	−	−	−
	10	150	184	+	−	−	−	−
	11	123	210	++	−	−	−	−
	12	133	220	+	−	+	Traces	−
	13	127	219		−	−	−	−

* According to Charconnet-Harding and Terroine, T. (1956).
† Minus sign indicates absence of xanthurenic acid. The number of crosses represents the intensity of the coloration due to xanthurenic acid.
‡ Minus sign indicates absence of the substance; plus sign indicates its presence.

3. Preventive Protective Power of Ascorbic Acid against the Endocrinian Symptoms of Pantothenic-acid Deficiency

(a) *Thymus*

The atrophy of the thymus appearing in pantothenic-acid deficiency is inhibited by ascorbic acid (Hundley and Ing, 1953).

(b) *Adrenals*

One of the specific symptoms of pantothenic-acid deficiency is a deep disorder of the adrenal cortex, which, from the histological point of view, shows finally in a degeneration of the cortex which vascularizes and necroses.

In the presence of ascorbic acid, however, the histological aspects of the adrenal cortex remain perfectly normal (Daft and Schwarz, 1952; Hundley and Ing, 1953; Barboriack and Krehl, 1957).

(c) *Genital Tract and Reproduction*

Pantothenic-acid deficiency seriously affects the genital tract of the rat.

In the male rat, there is a degeneration or decrease in the interstitial tissue, and spermatogenesis is disordered. But ascorbic acid succeeds only partially as a protection against these changes (Barboriack and Krehl, 1957).

In the female rat, pantothenic deficiency, like many vitamin deficiencies, upsets reproduction. Ascorbic acid corrects, to a certain extent, the disorders of gestation, as is shown in Table 5.3, taken from the work of Giroud *et al.* (1956).

TABLE 5.3*

Consequences of Administration of Ascorbic Acid on Accidents of Gestation in Rats Deficient in Pantothenic Acid

Treatment	No. of implantations whose evolution was studied	Percentage of foetus in relation to the number of implantations: Normal	Abnormal	Resorbed	Abnormal +Resorbed
Without ascorbic acid	196	21·9	22·9	55·1	
	Subcutaneous injection of ascorbic acid (500 mg/day)				78·0
With ascorbic acid	132	28·8	29·5	41·7	71·2

* According to Giroud *et al.* (1956).

This favourable activity of ascorbic acid is increased further when the diet of the pregnant rats contains a low dose of pantothenic acid (10 μg/day/rat, that is, 1/15th of the necessary dose) (Giroud *et al.*, 1956).

The association of pyrocatechine, in itself not very active, and ascorbic acid in a diet partially deficient in pantothenic acid, considerably improves the results still further, since, as Table 5.4 shows, the percentage of accidents then falls to 22 per cent, while it is 65 per cent in the group of non-supplemented rats in gestation. Pyrocatechine strengthens the action of ascorbic acid, probably by slowing down its oxidation *in vivo* (Lefebvre and Gero, 1957).

TABLE 5.4*

Consequences on the Gestation of Rats partially Deficient in Pantothenic Acid of the Administration of Pyrocatechine and Ascorbic Acid

Supplements administered	Group I without supplements	Group II 10 mg/day pyrocatechine	Group III 250 mg/day ascorbic acid	Group IV 10 mg/day pyrocatechine +250 mg/day ascorbic acid
Number of rats	9	9	8	9
Number of ovules implanted	93	94	88	100
per cent of foetus† — Normal‡	34·4	40·4	56·8	78·0
per cent of foetus† — Abnormal	26·8	28·6	23·9	15·0
per cent of foetus† — Resorbed	38·8	31·0	19·3	7·0
per cent of foetus† — Abnormal + resorbed	65·6	59·6	43·2	22·0

* According to Lefebvre and Gero (1957).
† In relation to the number of ovules implanted.
‡ Normal here means without morphological abnormalities

Finally, the average birth weight of young rats of females receiving neither pantothenic acid nor ascorbic acid was 3·7 g. The presence of the ascorbic acid in the deficient diet increased the birth weight of the young slightly (4·3 g). The presence of 100 μg/day/rat of pantothenic acid in the ration allowed the birth of larger litters composed of heavier young (4·6 g) (Everson *et al.*, 1954).

B. Curative Protective Power of Ascorbic Acid against Pantothenic-acid Deficiency

Administered to young rats during the 5th week of the pantothenic deficiency, that is, when this deficiency is well established, ascorbic acid improves the growth of the deficient rats (Fig. 5.4). This curative power is, however, limited, for the growth remains subnormal, and cutaneous troubles are not entirely eliminated; half the animals do not experience its influence (Terroine, T., 1959*b*).

Improvement in growth in the rat, as in the *L. arabinosus*; prolongation of the survival time; suppression, or considerable attenuation, of the metabolic and endocrinian disorders in the rat; these sum up the preventive protection exercised by ascorbic

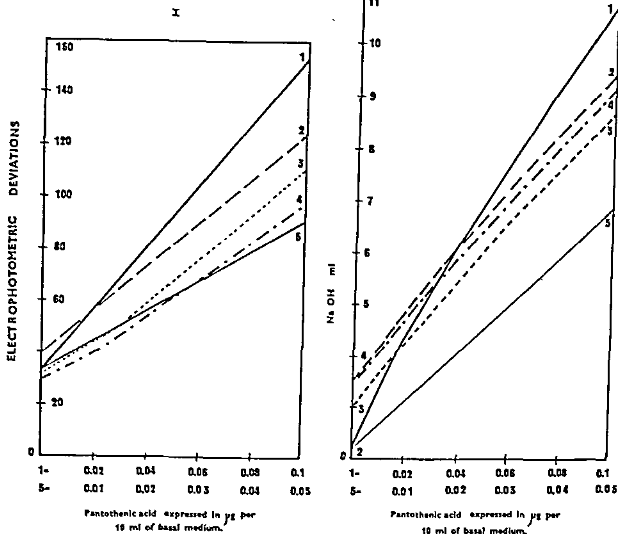


FIG. 5.5. INFLUENCE OF THE ADDITION OF DIFFERENT REDUCING AGENTS TO A MEDIUM LOW IN PANTOTHENIC ACID ON THE GROWTH OF *L. arabinosus* 17-5

The growth is measured by turbidimetry after 19-h incubation (I) or by acidimetry after 60-h incubation (II).

Curves 1: optimum concentration of pantothenic acid; curves 5: half optimum concentrations; the previous sub-optimum concentrations supplemented per 10 ml of basal medium with: 2 mg ascorbic or isoascorbic acid, curves 2; cysteine, 2 mg (I) or 5 mg (II), curves 3; glutathion 2 mg (I), 5 mg (II), curves 4 (according to *Terroine, T.*, 1958, 1959*a*)

the oxalacetic acid, thus contributing to the formation of citric acid. In pantothenic-acid deficiency, where this indispensable chemical action is slowed down and stopped, there is an unused accumulation of the generator of acetic acid, i.e. pyruvic acid.

Here again, the preventive protection of ascorbic acid against this serious manifestation of pantothenic-acid deficiency is absolutely perfect. This is shown by the determinations of blood pyruvic acid in newborn rats produced by females receiving one of the three following diets (Everson *et al.*, 1954):

	Blood Pyruvic acid *mg/100 ml*
Pantothenic acid deficient diet	2·79
Pantothenic acid deficient diet+2 per cent ascorbic acid	0 65
Diet containing 100 μg/day pantothenic acid	1·04

It should be noticed that the administration of *100 μg/day pantothenic acid* is slightly lower than the optimum, which is 150 μg/day.

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* According to Lefebvre and Gero (1957).
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Finally, the average birth weight of young rats of females receiving neither pantothenic acid nor ascorbic acid was 3·7 g. The presence of the ascorbic acid in the deficient diet increased the birth weight of the young slightly (4·3 g). The presence of 100 μg/day/rat of pantothenic acid in the ration allowed the birth of larger litters composed of heavier young (4·6 g) (Everson *et al.*, 1954).

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Improvement in growth in the rat, as in the *L. arabinosus*; prolongation of the survival time; suppression, or considerable attenuation, of the metabolic and endocrinian disorders in the rat; these sum up the preventive protection exercised by ascorbic

acid against pantothenic-acid deficiency. To this protection should also be added the by no means negligible curative activity.

Finally, it should be noted that substances such as glucuronolactone or gluconic acid also exercise a favourable action against pantothenic-acid deficiency in the rat (Hundley and Ing, 1953). This is quite understandable since these substances probably constitute stages in the synthesis of ascorbic acid (Mansvor-ul-Hassan and Lehninger, 1956; Grollman and Lehninger, 1957; Burns and Evans, 1956; Chatterjee *et al.*, 1960).

IV. PROTECTIVE POWER OF ASCORBIC ACID AGAINST BIOTIN DEFICIENCY

Preventive Protective Power of Ascorbic Acid against Biotin Deficiency

The dose of ascorbic acid administered to rats, based on that used by Dam in studies on vitamin-E deficiency, is from six to thirty times weaker—0·1 to 0·5 per cent of the ration—than that habitually and previously used. Perhaps this is the reason for certain restrictions in the substitutional role of ascorbic acid against biotin deficiency: *restrictions relating particularly to* the absence of the curative power (Terroine, T., 1955). It would, therefore, probably be interesting to repeat the following studies using this time the maximum doses of ascorbic acid habitually used in the previous research.

1. Preventive Protective Power of Ascorbic Acid against the Apparent Symptoms of Biotin Deficiency

(a) *Growth*

As mentioned above, the body weight of rats *deficient in biotin and receiving 0·1 to 0·5 per cent* ascorbic acid is only slightly improved compared with that of the group deficient but not receiving vitamin C (Terroine, T., 1954).

On the other hand, ascorbic acid exercises an *important substitutional activity, but not a complete one* in regard to *L. arabinosus* 17/5 placed in a medium partially deficient in biotin. This beneficial effect of ascorbic acid upon the growth of this micro-organism is quite independent of the period of incubation, whether long or short (Fig. 5.6) (Terroine, T., 1958, 1959*a*).

(b) *Appetite*

Appetite is, like growth, only slightly stimulated by a relatively moderate addition of ascorbic acid to the diet of *biotin deficiency in the rat (Terroine, T., 1954).*

(c) *Apparent Symptoms Characteristic of Biotin Deficiency*

The preventive protection of ascorbic acid is exercised particularly on the apparent clinical symptoms characteristic of *biotin deficiency: (1) a general alopecia*, frequently, of the whole body. Before falling, the hair ceases to be white, shiny and straight and becomes yellowish, dull and curly. The epidermis is covered with a brownish scurf; (2) *oedema* and an *irritation of the eyelids*, making red "spectacles" around the eyes; (3) *a typical disorder of gait*, an excessive raising of the sacral-lumbar region of the vertebral column, giving the appearance and gait known as "kangaroo".

On the 92nd day of biotin deficiency, all the animals deprived of this vitamin showed a more or less pronounced alopecia, 60 per cent of them had a bad pelt, 90 per cent had "spectacle eyes" and 50 per cent had the abnormal "kangaroo" gait. At the same time, alopecia was avoided in 70 per cent of the deficient subjects receiving ascorbic acid; the coat of 90 per cent of these was in perfect condition, and none had "spectacle eyes" or the abnormal "kangaroo" gait (Terroine, T., 1954).

(d) *Survival Time*

On the 92nd day of biotin deficiency, all the animals *deprived of ascorbic acid* were dead, while 70 per cent of those receiving it were still alive. It was only by the 125th day that the mortality rate rose to 60 per cent. It is also worth noting that half the deficient rats treated with ascorbic acid showed at the time of death no apparent symptom of biotin deficiency. It seems, therefore, that with this deficiency the processes which determine specific, apparent characteristic symptoms should be dissociated from those causing death; the first, and not the second, being those which can be eliminated by the administration of heavy doses of ascorbic acid (Terroine, T., 1954).

2. Preventive Protective Power of Ascorbic Acid against the Characteristic Metabolic Symptoms of Biotin Deficiency

Urinary Excretion of Ammonia

On the metabolic plane, biotin deficiency is characterized, *in particular, by a very marked increase* in urinary excretion of ammonia (Terroine, T. and Rombauts, 1952). This trouble is the secondary consequence of an acid intoxication produced by a strong pyruvicemia (Terroine, T., 1956).

Ascorbic acid slightly attenuates this large excretion of ammonia in the urine; while the urinary excretion of ammoniacal nitrogen reaches 6·40 mg/day in the rat with *slight biotin deficiency, it drops to*

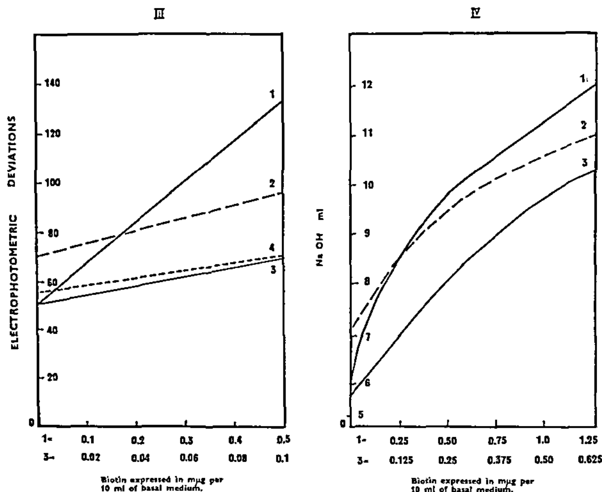


Fig. 5.6. Influence of the Addition of Different Reducing Agents to a Medium Low in Biotin on the Growth of *L. arabinosus* 17-5

The growth is measured by turbidimetry after 19-h incubation (III) or by acidimetry after 60-h incubation (IV).

Curves 1: optimum concentration in biotin· curve 3· 1/5 or half optimum concentrations; curves 2 and 4: the previous sub-optimum concentrations respectively supplemented with ascorbic or isoascorbic acid (curves 2) or with cysteine (curve 4) at the rate of 2 mg/ml of basal medium (according to *Terroine, T.*, 1958, 1959*a*).

5·40 mg with the addition of ascorbic acid to the deficient diet. The normal value is 3·40 mg (Terroine, T., 1954).

In conclusion, it can be said that the protective action of ascorbic acid is good but not perfect; there is an absence of curative power and preventive power for certain characteristic symptoms of biotin deficiency. As for the previous studies, it would be profitable to extend these observations to other animal species.

V. PROTECTIVE POWER OF ASCORBIC ACID AGAINST FOLIC ACID DEFICIENCY

Curative Protective Power of Ascorbic Acid against Folic-acid Deficiency

Contrary to the previous studies, only the curative action of ascorbic acid against folic-acid deficiency in the rat, and in the chick, has been considered here.

(a) *Growth*

Undeniably, in the rat and the chick, ascorbic acid provokes a certain revival in growth which was slowed down because of folic-acid deficiency. Thus, after 15 days' preliminary folic-acid deficiency the gain in body

acid against pantothenic-acid deficiency. To this protection should also be added the by no means negligible curative activity.

Finally, it should be noted that substances such as glucuronolactone or gluconic acid also exercise a favourable action against pantothenic-acid deficiency in the rat (Hundley and Ing, 1953). This is quite understandable since these substances probably *constitute stages in the synthesis of ascorbic acid* (Mansvor-ul-Hassan and Lehninger, 1956; Grollman and Lehninger, 1957; Burns and Evans, 1956; Chatterjee *et al.*, 1960).

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Appetite is, like growth, only slightly stimulated by a relatively moderate addition of ascorbic acid to the diet of biotin deficiency in the rat (Terroine, T., 1954).

(c) *Apparent Symptoms Characteristic of Biotin Deficiency*

The preventive protection of ascorbic acid is exercised particularly on the apparent clinical symptoms characteristic of biotin deficiency: (1) a general *alopecia*, frequently, of the whole body. Before falling, the hair ceases to be white, shiny and straight and becomes yellowish, dull and curly. The epidermis is covered with a brownish scurf; (2) *oedema* and an irritation of the eyelids, making red "spectacles" around the eyes; (3) *a typical disorder of gait*, an excessive raising of the sacral-lumbar region of the vertebral column, giving the appearance and gait known as "kangaroo".

On the 92nd day of biotin deficiency, all the animals deprived of this vitamin showed a more or less pronounced alopecia, 60 per cent of them had a bad pelt, 90 per cent had "spectacle eyes" and 50 per cent had the abnormal "kangaroo" gait. At the same time, *alopecia was avoided in 70 per cent of the deficient* subjects receiving ascorbic acid; the coat of 90 per cent of these was in perfect condition, and none had *"spectacle eyes" or the abnormal "kangaroo" gait* (Terroine, T., 1954).

(d) *Survival Time*

On the 92nd day of biotin deficiency, all the animals deprived of ascorbic acid were dead, while 70 per cent of those receiving it were still alive. It was only by the 125th day that the mortality rate rose to 60 per cent. It is also worth noting that half the deficient rats treated with ascorbic acid showed at the time of death no apparent symptom of biotin deficiency. It seems, therefore, that with this deficiency the processes which determine specific, apparent characteristic symptoms should be dissociated from those causing death; the first, and not the second, being those which can be eliminated by the administration of heavy doses of ascorbic acid (Terroine, T., 1954).

2. Preventive Protective Power of Ascorbic Acid against the Characteristic Metabolic Symptoms of Biotin Deficiency

Urinary Excretion of Ammonia

On the metabolic plane, biotin deficiency is characterized, in particular, by a very marked increase in urinary excretion of ammonia (Terroine, T. and Rombauts, 1952). This trouble is the secondary consequence of an acid intoxication produced by a strong pyruvicemia (Terroine, T., 1956).

Ascorbic acid slightly attenuates this large excretion of ammonia in the urine; while the urinary *excretion of ammoniacal nitrogen reaches 6·40 mg/day* in the rat with slight biotin deficiency, it drops to

serious symptom, which strikes almost all the non-supplemented animals (Zacharias *et al.*, 1950; Dam *et al.*, 1948).

An interesting fact is that neither thiamine nor riboflavin, nor nicotinic acid can be substituted for the beneficial action of ascorbic acid (Zacharias *et al.*, 1950).

The depigmentation of the incisors of the rat, due, probably, to the disappearance of a ferric phosphate (Dam, 1957), is, on the other hand, only very slightly attenuated by ascorbic acid (Granados *et al.*, 1949).

Muscular dystrophy, with atrophy of the muscles and inco-ordination of gait, is, in the guinea-pig, considerably reduced by ascorbic acid. After eight weeks of vitamin-E deficiency, all the animals were struck with serious dystrophy which appeared in only 19 per cent of the animals receiving 2 mg/day/ascorbic acid, and 62 per cent of the subjects in this last group escaped the trouble entirely (Farmer *et al.*, 1950).

But the protective activity of ascorbic acid against muscular dystrophy is insignificant in the calf (Blaxter *et al.*, 1953) and nil in the chick (Dam, 1957).

Anaemia caused, in the rat, by a diet deprived of iron and low in vitamin E, is combated as effectively with addition of iron–vitamin E, as with a supplement of iron–ascorbic acid. But the haemoglobin regeneration is optimum with the simultaneous administration of iron, vitamin E and ascorbic acid (Greenberg *et al.*, 1957).

2. Preventive Protective Power of Ascorbic Acid against the Metabolic Symptoms of Vitamin-E Deficiency

Peroxidation of Lipids

The principal biochemical role of vitamin E—but perhaps not the exclusive one as discussed by Dam (1953, 1957)—is to act as an anti-oxidant. Indeed, the association of vitamin-E deficiency and a strong surcharge of highly unsaturated lipids is indispensable or necessary for the appearance of the symptoms enumerated above: exudative diathesis, encephalomalacia, depigmentation of the incisors of the rat, muscular dystrophy (Dam, 1957). In the absence of the anti-oxidant, which is the vitamin E, these lipids engender peroxides whose appearance coincides with that of the clinical symptoms (Dam, 1957).

Although the search for peroxides in the lipids of brains of chicks has, hitherto, been unsuccessful, encephalomalacia is, nevertheless, probably a variant of the same metabolic trouble of the lipids (Dam, 1957).

Table 5.5 shows the great effectiveness of ascorbic acid in combating the peroxidation of the lipids whose intensity is measured directly by chemical means and whose indirect indication is the yellowish brown coloration (Dam and Granados, 1945).

TABLE 5.5

Consequences of the Administration of Ascorbic Acid on the Peroxidation of Fat in Vitamin-E Deficiency

Nature of diets	Rat		Chick
	I Index of peroxidation of total fat after 10 weeks	II Coloration of lumbar fat after 10 weeks	Index of peroxidation of total fat after 10 weeks
Diet deficient in E	160	5 = dark brown yellow	21
Diet deficient in E +0·5 g per cent ascorbic acid	79	3 = dark yellow	0·2
Diet supplemented with 0·01 to 0·02 g per cent E	9·9	0 = colourless Scale of coloration from 0 to 5	0·8
References	Granados *et al.* (1949)		Dam *et al.* (1951)

The preventive action of ascorbic acid against vitamin-E deficiency is thus very considerable, but it is not complete, for certain symptoms such as muscular dystrophy in the calf, and the depigmentation of the incisors of the rat, are hardly affected. Others are not affected at all: the brown coloration of the uterus of the rat (Moore *et al.*, 1954), the depletion of vitamin A of the liver, and the massive necrosis of this organ (Dam, 1957).

It may be added, in conclusion, that the sole attempt to show a curative power of ascorbic acid against vitamin-E deficiency gave a negative result.

The administration of a single dose of ascorbic acid to rats previously heavily deficient in vitamin E in no way reduces the peroxidation index of their abdominal fat (Hanson *et al.*, 1944). Could it be that the favourable influence of ascorbic acid is solely preventive, or do the negative results of the authors depend on the dose of ascorbic acid and the strain of rats used (Dam *et al.*, 1948)?

VIII. PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN A DEFICIENCY

The protection offered by ascorbic acid is far from unanimously recognized.

Recent facts confirming the existence of this protection are grouped in Table 5.6, where it can be seen

weight of three groups of chicks—at the end of a new period of observation of 15 days—was established comparatively as follows: it was 19 g for the group kept on a strict folic-acid deficient diet, and rose to 35 g when 100 mg ascorbic acid for 100 g of ration was introduced into the diet; but this increase was slight compared with that of 140 g acquired by the group of chicks which received the optimum dose of 500 μg folic acid per 100 g diet (Dietrich *et al.*, 1949). Moreover, the average weight of the rats, which, after seven weeks of folic-acid deficiency was 100 g, rose immediately and regularly up to 130 g after three weeks' supplementation of 2 mg per day of ascorbic acid (Johnson and Dana, 1948).

(b) *Survival Time*

In this same experiment on rats, ascorbic acid did not clearly give any resistance to the mortality of the animals.

(c) *Haemorrhage*

On the other hand, it cured definitely the apparent "clinical" symptoms of folic-acid deficiency shown by haemorrhage of the snout and paws.

(d) *Anaemia*

With these same rats, the introduction of ascorbic acid, after seven weeks' folic-acid deficiency, considerably improved the blood picture; after 21 days of supplementation in ascorbic acid, the white blood corpuscles rose from 3,920/mm^3 to 7,210 and the granulocytes multiplied more than ten times, from 182/mm^3 to 1,956. But the process of maturation of the red blood corpuscles remained paralysed and only the administration of folic acid lifted this inhibition (Johnson and Dana, 1948).

In conclusion, the replacement exercised by ascorbic acid against folic-acid deficiency appears to be active and widespread.

VI. PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN-B_{12} DEFICIENCY

Much remains to be done in this field, since the studies undertaken up to now have concerned only micro-organisms. Several lactobacilli, in particular, can grow in a medium deprived of B_{12}, which is normally necessary to them, if this medium is supplemented by ascorbic acid (Welch and Wilson, 1949; Shive *et al.*, 1948; Koditchek *et al.*, 1949; Kipay *et al.*, 1950).

VII. PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN E DEFICIENCY

Preventive Protective Power of Ascorbic Acid against Vitamin-E Deficiency

1. Preventive Protective Power of Ascorbic Acid against the Apparent Symptoms of Vitamin-E Deficiency

(a) *Growth*

A dose of ascorbic acid—0·5 per cent of the E-deficient diet—is incapable of stimulating the growth of the rat deprived of vitamin E (Granados *et al.*, 1949), but it improves the growth of the deficient chick, either slightly (Dam *et al.*, 1951) or, sometimes, very considerably. This was so when, in 24 days, the body weight of chicks with vitamin-E deficiency increased from 35 to 80 g, while with a supplement of ascorbic acid it increased to 250 g in 56 days (Dam *et al.*, 1948). The diversity of the reaction of ascorbic acid to different species with a vitamin-E deficiency naturally makes it more difficult to understand the mechanism of its action.

(b) *Characteristic Symptoms of Vitamin-E Deficiency*

The exudative diathesis affects, particularly, the adipose tissue, but also the muscle and the skin. The trouble begins with a diffuse haemorrhage and continues with an exudation of plasma from the capillaries. The exudate is generally a green colour by reason of the degradation of the haemoglobin. The ventral side of the animal is swollen. Exudation diathesis can be mortal.

Ascorbic acid exercises an excellent preventive protection against this symptom of vitamin-E deficiency. After five weeks' treatment, 7/9 of the chicks not receiving ascorbic acid suffered from diathesis, while only 2/9 of the animals supplemented with ascorbic acid showed this symptom (Dam *et al.*, 1951). Sometimes ascorbic acid even completely inhibits the appearance of exudative diathesis (Dam *et al.*, 1948). The protective influence of ascorbic acid is already considerable with doses of 0·1 or even 0·06 per cent (Dam *et al.*, 1948, 1951).

Encephalomalacia of the chick is a disorder of the brain, producing ataxia, retraction of the head, paralysis of the spastic type, and finally death. Here again, ascorbic acid—whether at the rate of 5 mg/day/animal, or 0·5 per cent of the ration—inhibits almost completely the appearance of this

serious symptom, which strikes almost all the non-supplemented animals (Zacharias *et al.*, 1950; Dam *et al.*, 1948).

An interesting fact is that neither thiamine nor riboflavin, nor nicotinic acid can be substituted for the beneficial action of ascorbic acid (Zacharias *et al.*, 1950).

The depigmentation of the incisors of the rat, due, probably, to the disappearance of a ferric phosphate (Dam, 1957), is, on the other hand, only very slightly attenuated by ascorbic acid (Granados *et al.*, 1949).

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But the protective activity of ascorbic acid against muscular dystrophy is insignificant in the calf (Blaxter *et al.*, 1953) and nil in the chick (Dam, 1957).

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2. Preventive Protective Power of Ascorbic Acid against the Metabolic Symptoms of Vitamin-E Deficiency

Peroxidation of Lipids

The principal biochemical role of vitamin E—but perhaps not the exclusive one as discussed by Dam (1953, 1957)—is to act as an anti-oxidant. Indeed, the association of vitamin-E deficiency and a strong surcharge of highly unsaturated lipids is indispensable or necessary for the appearance of the symptoms enumerated above: exudative diathesis, encephalomalacia, depigmentation of the incisors of the rat, muscular dystrophy (Dam, 1957). In the absence of the anti-oxidant, which is the vitamin E, these lipids engender peroxides whose appearance coincides with that of the clinical symptoms (Dam, 1957).

Although the search for peroxides in the lipids of brains of chicks has, hitherto, been unsuccessful, encephalomalacia is, nevertheless, probably a variant of the same metabolic trouble of the lipids (Dam, 1957).

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Diet supplemented with 0 01 to 0·02 g per cent E	9 9	0 = colourless Scale of coloration from 0 to 5	0 8
References	Granados *et al.* (1949)		Dam *et al.* (1951)

The preventive action of ascorbic acid against vitamin-E deficiency is thus very considerable, but it is not complete, for certain symptoms such as muscular dystrophy in the calf, and the depigmentation of the incisors of the rat, are hardly affected. Others are not affected at all: the brown coloration of the uterus of the rat (Moore *et al.*, 1954), the depletion of vitamin A of the liver, and the massive necrosis of this organ (Dam, 1957).

It may be added, in conclusion, that the sole attempt to show a curative power of ascorbic acid against vitamin-E deficiency gave a negative result.

The administration of a single dose of ascorbic acid to rats previously heavily deficient in vitamin E in no way reduces the peroxidation index of their abdominal fat (Hanson *et al.*, 1944). Could it be that the favourable influence of ascorbic acid is solely preventive, or do the negative results of the authors depend on the dose of ascorbic acid and the strain of rats used (Dam *et al.*, 1948)?

VIII. PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN A DEFICIENCY

The protection offered by ascorbic acid is far from unanimously recognized.

Recent facts confirming the existence of this protection are grouped in Table 5.6, where it can be seen

TABLE 5.6

Consequences of the Administration of Ascorbic Acid on the Symptoms of Vitamin-A Deficiency

Nature of diets	Species studied				
	Young foxes		Young rats		
Basis of alimentation	Powdered milk, soya, etc. *Vitamin:Yeast*		Casein, saccharose, corn oil, *salt mixture, synthetic vitamins*		
	Growth	Apparent symptoms of A deficiency	Body weight after 35 days diet (g)	Survival time	Apparent symptoms of A deficiency
Diet deficient in A	bad	in 38 p. 100 of animals	175	40 days	+
Diet deficient in A+ascorbic acid*	no improvement	suppression notably of troubles of gait	229	all alive on the 80th day of treatment	0 xerophthalmia 0 symptom
Diet supplemented with 1,200 I.U.A. every 8 days			343		
References	Basset *et al.* (1948)		Mayer and Krehl (1948)		

* 4 mg/100 g of diet (Basset *et al.*, 1948). *100 mg/100 g of diet (Mayer et al., 1948).*

that the administration of ascorbic acid gives an appreciable improvement of growth in the rat.

The survival time in the rat is remarkably prolonged.

Finally, certain apparent symptoms of vitamin-A deficiency are completely suppressed in the fox and the rat. As opposed to the above results there are others entirely negative.

They concern solely the young rat with vitamin-A deficiency, where this time the addition of ascorbic acid neither improved the growth (Sharmann, 1949; Guerrant, 1948), nor the general health (Guerrant, 1948).

It is thus not possible to pronounce positively between these two groups of incompatible results. More experiments are needed before the debate can be concluded; they must show whether the conflicting results depend on the doses of ascorbic acid administered, on the *varieties of animal species used*, and on *the nature of diets.*

At the present stage of our knowledge the preventive action of ascorbic acid in vitamin-A deficiency is thus still in question.

On the other hand, and on the basis of a single experiment, ascorbic acid seems to have no curative influence with regard to vitamin-A deficiency; the administration to six young calves of 2 g/day ascorbic acid in subcutaneous injections during fourteen days did not halt the symptoms of this deficiency, which, naturally, the addition of vitamin A succeeded in doing (Helmbolt *et al.*, 1953).

Finally, ascorbic acid often affords considerable protection against vitamin deficiencies whose manifestations are extremely different. That is, vitamins with apparently very distinct physiological properties may be reduced, in a way, to a "common denominator," ascorbic acid.

These very large vitamin interrelations can probably *be explained on the basis of a common feature* of certain physiological and biochemical functions.

The use of *ascorbic acid makes it possible*—there are many examples—to dissociate from one another certain vitamin activities, since, in a given deficiency, *vitamin C ensures the efficient working of some* activities and not others.

But the favourable action of ascorbic acid is not connected systematically with the same function: it does not automatically improve the body weight of deficient animals. *The fall in weight, which is observed in all deficiencies, reveals* complex processes not exclusively connected with the functions of ascorbic acid.

Finally, the substitutional ability of ascorbic acid with regard to vitamins is exercised with heavy doses—from 0·1 g to 3 g per cent of the ration generally—which are out of comparison with the physiological needs for vitamins.

PART TWO: CHARACTERS AND MECHANISMS OF THE PROTECTIVE POWER OF ASCORBIC ACID AGAINST VITAMIN DEFICIENCIES

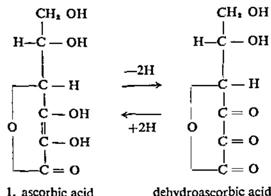
Five arguments, of very unequal bearing, may be invoked in attempting to understand the remarkable substitutional property of ascorbic acid against various vitamin deficiencies. These are as follows:

1. The suppression, by ascorbic acid, of the secondary deficiency in vitamin C induced by divers vitamin deficiencies.
2. The vitamin-sparing action by ascorbic acid.
3. The functional substitution of ascorbic acid for missing vitamins.
4. The anti-infectious property of ascorbic acid.
5. The antitoxic property of ascorbic acid.

These processes do not exclude one another but can function simultaneously.

The intimate mechanism of these processes, when it is sought, reveals almost always a single explanation: the participation of ascorbic acid in oxydo-reduction operations.

Indeed, by its structure and, particularly, its dienol group in position 2-3, ascorbic acid possesses two labile hydrogens making, *a priori* the molecule a perfect hydrogen carrier, according to the following equation:



1. ascorbic acid dehydroascorbic acid

Now, while *in vitro*, there is no doubt about this equation, the whole problem lies in whether it can reasonably be transposed *in vivo*. To make ascorbic acid a veritable respiratory catalyst one must be able to show the reality of the existence and the intimate mechanism of the reversible reaction. 1. ascorbic acid ⇆dehydroascorbic acid.

Working on pea seeds, Mapson and Moustafa (1956) were able to show the reality of the respiratory catalyst role of ascorbic acid in vegetable metabolism.

In animal metabolism, the works of Kersten *et al.* (1956) seem to show the participation of ascorbic acid in the transport of hydrogen during the oxidation of the diphosphopyridine nucleotide by the adrenal mitochondria. But the dehydroascorbic acid does not appear in this reaction.

Supplementary details about the exact mechanism of the reaction are obviously necessary. It would also be necessary to know if the role of ascorbic acid in the hydrogen transport, thus shown, is general or applies only to the adrenal glands.

According to Radsma (1957) also, working on a homogenate of liver, ascorbic acid must act as a respiratory catalyst and facilitate the oxidation of the diphosphopyridine nucleotide.

However, before being able to consider, with certainty, ascorbic acid as a carrier of hydrogen *in vivo* in animal metabolism, there is no doubt that it can be regarded, in any case, as a donor of rapidly usable labile hydrogen. By this power of easily yielding hydrogen, ascorbic acid may, in this way, play the role of an antioxidant in animal metabolism (Meiklejohn, 1953; Mapson, 1954).

Having pointed out the general principles of the substitutional activity of ascorbic acid in vitamin deficiencies we can now determine the modes of application of these principles to a particular well-defined deficiency.

In reality, however, as will be seen, it is rarely that a systematic investigation shows completely the mechanism of the protective role of ascorbic acid against a particular vitamin deficiency. There will, therefore, be many gaps to be filled in before we can have a complete picture of the polyvalent substitutional activity of ascorbic acid in vitamin deficiencies.

I. SUPPRESSION BY ASCORBIC ACID OF SECONDARY DEFICIENCY IN VITAMIN C INDUCED BY VARIOUS VITAMIN DEFICIENCIES

Is not the protective action of ascorbic acid against various vitamin deficiencies due, at least partly, to the fact that it combats a secondary deficiency in vitamin C induced by these various deficiencies? In answer to

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It is thus not possible to pronounce positively between these two groups of incompatible results. More experiments are needed before the debate can be concluded; *they must show whether the conflicting* results depend on the doses of ascorbic acid administered, on the varieties of animal species used, and on the nature of diets.

At the present stage of our knowledge the preventive action of ascorbic acid in vitamin-A deficiency is thus still in question.

On the other hand, and on the basis of a single experiment, ascorbic *acid seems to have no curative* influence with regard to vitamin-A deficiency; the administration to six young calves of 2 g/day ascorbic acid in subcutaneous injections during fourteen days did not halt the symptoms of this deficiency, which, naturally, the addition of vitamin A succeeded in doing (Helmbolt *et al.*, 1953).

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These very large vitamin interrelations can probably be explained on the basis of a common feature of certain physiological and biochemical functions.

The use of ascorbic acid makes it possible—there *are many examples*—to dissociate from one another certain vitamin activities, since, in a given deficiency, vitamin C ensures the efficient working of some *activities and not others.*

But the favourable action of ascorbic acid is not connected systematically with the same function: it does not automatically improve the body weight of deficient animals. The fall in weight, which is observed in all deficiencies, reveals complex processes not exclusively connected with the functions of ascorbic acid.

Moreover, as the deficiency modifies simultaneously the weight of the organs and the total weight of the body, one must determine not the ascorbic acid content per g of tissue, but its content in the whole organ and brought up to a 100 g of the weight of the body. In these conditions it has been calculated that at the pre-mortal stage of biotin deficiency, the ascorbic acid capital is only diminished by 17 per cent (Terroine, T., 1954).

Estimated by taking into account these two observations, the secondary deficiency in ascorbic acid, provoked by a given vitamin deficiency, is often less important than appears at first sight.

In any case, when it exists, as in the deficiencies in thiamine, riboflavin, folic acid, etc., what is the cause of this secondary deficiency in ascorbic acid? Is it decrease in the ability to synthetize this factor, disorders of its renal excretion, or increase in its consumption?

Two indications make it possible to determine whether the deficiency in a given vitamin weakens the rat's ability to synthetize ascorbic acid. If this is so, the ascorbic acid content must decrease, not only in the tissues, but also in the urine; if, indeed, a fall in in the concentration of the tissues in ascorbic acid corresponded to a rise in urinary elimination, this would suggest a disorder of renal elimination of vitamin C, characterized by an excessive excretion of the latter. It is on the basis of this indirect indication that Schwartz and Williams (1952), on the one hand, and Boyer *et al.* (1942) on the other, admit, respectively, that the deficiency in folic acid or in vitamin A produces a disorder of the synthesis of ascorbic acid in the rat (Table 9.7).

The second indication consists in using chloretone, which has the property of stimulating the synthesis of ascorbic acid. If a given deficiency affects this specifically, the stimulating influence of the chloretone would be inhibited, or at least reduced. Now, the activity of chloretone in no way slows down in folic-acid deficiency (Lahiri and Banergee, 1956) nor does it in vitamin-A deficiency (Mapson and Walker, 1948). Contrary to previous deductions, these two vitamin deficiencies do not then seem to involve any unfavourable effect on the synthesis of ascorbic acid in the rat.

On the other hand, it is by using this same test with chloretone that Roy *et al.* (1946) observed an inhibition of the synthesis of ascorbic acid in thiamine and riboflavin deficiency. For Ekman and Strombeck (1949) the participation of riboflavin in the synthesis of ascorbic acid can probably be explained by its intervention in the glucidic metabolism; ascorbic acid being synthetized from the glucids.

As for the hypothesis of an increase in consumption of ascorbic acid by the rat with a deficiency in a given vitamin, it has not been verified.

On the whole, it can be said that the administration of a heavy dose of ascorbic acid during various vitamin deficiencies may sometimes suppress a secondary deficiency in this factor and, in consequence, improve the general state of the animal. But this suppression of a secondary deficiency in ascorbic acid is not a sufficient explanation to understand the substitutional role of ascorbic acid in vitamin deficiencies.

II. VITAMIN SPARING ACTION BY ASCORBIC ACID

The sparing action upon a given vitamin in a diet deprived of an exterior addition of this vitamin may be ascribable to two processes working separately or jointly.

1. An intervention in the synthesis of the vitamin; this intervention may itself be either a direct participation in this synthesis, or an indirect participation by stimulation of the intestinal microflora able to carry out this synthesis.

2. A limitation or a total stoppage of the using up of the vitamin in the organism becoming deficient; this limitation, or stoppage, affecting the vitamin which is still present, either in the organism becoming deficient or in the deficient diet, or in these two possibilities at the same time.

In fact ascorbic acid can exercise a vitamin-sparing activity by these two different processes.

Finally, ascorbic acid achieves, to different degrees, the sparing action upon several vitamins by one or some mechanisms as yet undetermined.

A. Vitamin-sparing Action by Participation of Ascorbic Acid in the Synthesis of Vitamins

1. Stimulation of the Synthesis of Thiamine by Ascorbic Acid

The only positive facts concern a study on micro-organisms. In the absence of thiamine, but in the presence of ascorbic acid, the *L. fermenti* 36 still retains 50 per cent of thiamine; moreover, the association of ascorbic acid and thiamine increases from 10 to 15 per cent the content of thiamine in this same bacillus. Without giving the intimate mechanism of this effect of ascorbic acid, the authors think that the ascorbic acid participates in the synthesis of the thiamine (Fang and Butts, 1953). If this is so, the favourable influence, previously mentioned, of ascorbic acid on the growth of *L. fermenti* 36 in a medium low in thiamine would thus be easily comprehensible.

Examining young rats after four weeks' thiamine deficiency, Scott and Griffith (1957) noticed a liver

TABLE 5.7

Consequences of the Deficiency in Various Vitamins on the Concentration of Ascorbic Acid in the Rat

Vitamins subtracted	Nature of diets	Liver mg/100 g of fresh tissue	Adrenals mg/100 g of fresh tissue	Lung mg/100 g of fresh tissue	Kidney mg/100 g of fresh tissue	Blood mg/100 ml	Urine
Thiamine	Control diet restricted	25 21·2	361	27	16 20 8		
	Diet deficient in *thiamine*	*21* *17·6*	*319*	20	13 15·2		
	Difference per cent	—19	—13	—35	—23		
	References	(a) (b)	(a)	(a)	(a) (b)		
Riboflavin	Control diet	26(R) 14·6(AL)	395(R)	24(R)	16 (R)		
	Diet deficient in riboflavin	18·7 14·1	383	17	11		0
	Difference per cent	—14	—*8*	—*41*	—*45*		
	References	(a) (c)	(a)	(a)	(a)		(d)
Pantothenic *acid*	Control diet	22·5(AL)	326(AL)				mg/100 g of the body weight 2 0
	Diet deficient in pantothenic acid	23·8	337				0·37
	Difference per cent	+5	+15·6				
	References	(e)	(e)				(f)
Biotin	Control diet	40(AL)	504(AL)				
	Diet deficient in biotin	30	*455*				
	Difference per cent	—25	—10				
	References	(g)	(g)				
Folic acid	*Control diet*	*31*(AL) *31·2*(R)	413(R)		20 9(R)		mg/rat/day 1·21
	Diet deficient in folic acid	19 23 8	273		15·3		0 63
	References	(h) (i)	(i)		(i)		(h)
A	Control diet	34·5(AL) 22(R) 22(R)	355(AL) 368(R) 360(R)			1·61(AL) 0 80(R)	mg/kg animal 0 49(AL)
	Diet deficient in A	15·6 21 19	212 352 330			0 7 0 38	0 19
	Difference per cent	—4·5	—4 5				
	References	(j) (a) (k)	(g) (a) (k)			(j) (k)	(l)

AL: *ad lib.* diet. R: *restricted* diet.
References: (a) Sure *et al.* (1939)
(b) Bhattacharya (1937)
(c) Ekman and Strombeck (1949)
(d) Maslenikowa (1950)
(e) Gero and Lefebvre-Boisselot (1957)
(f) Dumm and Ralli (1949)
(g) *Terroine*, T. (1954)
(h) Schwartz and Williams (1952)
(i) Lahiri and Banerjee (1956)
(j) Mayer and Krehl (1948)
(k) Mapson and Walker (1948)
(l) Boyer *et al.* (1942)

this question, Table 5.7 groups, as an example, the information relative to the consequences of various vitamin deficiencies on the concentration of ascorbic acid in the rat, the animal which has been most often used to show the substitutive power of ascorbic acid. The observations on the rat are particularly interesting moreover, since this animal can synthetize, in normal conditions, the quantities of ascorbic acid which are necessary to it. Similar observations to those carried out on the rat have been made on the pigeon (Lecoq and Findler, 1953), the chick (Szepsenwof, 1951), the calf (Boyer *et al.*, 1942), etc.

From an examination of Table 5.7 it is seen that the repercussions of the various vitamin deficiencies on the ascorbic acid content are far from identical. Thus, the secondary deficiency in ascorbic acid is nil in pantothenic-acid deficiency, very considerable in vitamin-B_2 or folic-acid deficiency, moderate in thiamine deficiency or in biotin deficiency, disputed in vitamin-A deficiency.

To be able to affirm definitely the existence of a secondary deficiency in ascorbic acid, one must compare the vitamin-C content of a subject deficient in a given vitamin, with a control, not fed *ad lib.*, but in a restricted quantity, equal to that ingested by the deficient subject.

Thus, on eliminating the appetite factor it can be seen that the vitamin-C deficiency determines, often, in the liver and the adrenals in particular, only an insignificant secondary deficiency (Table 5.7).

weeks, to one-tenth of its normal value (Terroine, T., 1957*a*).

Perhaps it might be possible to suggest as a hypothesis that in the presence of ascorbic acid the organism makes better use of the traces of the thiamine that it still has at its disposal (Le Breton, verbal communication).

Thus, in any case, it is not by an economy of thiamine, whatever its mechanism, that one can explain, in the rat, the protective role of ascorbic acid against the apparent, metabolic and endocrinian symptoms of vitamin-B_1 deficiency. It remains to be seen if this is also true for the pigeon deficient in thiamine, and sensitive to the protective action of ascorbic acid.

2. Stimulation of the Synthesis of Folic and Folinic Acids by Ascorbic Acid

Ascorbic acid exercises only a slight sparing action upon folic acid; as is shown by Table 5.9, its activity is comparable with that of the decidedly sub-optimum dose of 50 μg folic acid per 100 g of the ration (Dietrich *et al.*, 1949).

*TABLE 5.9**

Consequences of the Administration of Ascorbic Acid on the Folic Acid Content of the Liver of the Chicks Deficient in Folic Acid

Nature of diets	Folic acid content per total liver, in μg
Chick deficient in folic acid	2·82
Chick deficient in folic acid + 100 mg per cent ascorbic acid	4·65
Chick receiving 50 μg folic acid/100 g of ration	4·02
Chick receiving 500 μg folic acid/100 g of ration	17·97

* According to Dietrich, Nichol *et al.*, 1949.

On the other hand, it is possible that ascorbic acid stimulates the conversion of folic acid into folinic acid. The latter, still called the *citrovorum factor* because it is a growth factor of the *Leuconostoc citrovorum*, is one of the biologically active forms of folic acid. It is N-5-formyltetrahydropteroyl-glutamic acid. It seems that ascorbic acid probably participates in its formation from folic acid by yielding to the latter its labile hydrogens (Cheldelin and King, 1954). Chicks' or rats' liver slices put together with folic acid will convert this substance into folinic acid up to 25 per cent on condition that the medium contains ascorbic acid (Nichol, 1953; Nichol and Welch, 1950). This new property of ascorbic acid, however, cannot explain its sparing action upon folic acid, since it can work from the latter only as a substrate. At the most, ascorbic acid can be considered to contribute in limiting the depletion of folinic acid in an organism during folic-acid deficiency.

B. Vitamin-sparing Action to Suppress Ascorbic Acid by Inhibition of the Vitamin's Destruction

Ascorbic acid seems to exercise this mode of protection, only possibly in B_{12} deficiency, probably in folic-acid deficiency, and certainly in E deficiency.

1. Inhibition of Destruction of Folinic Acid by Ascorbic Acid

Nichol (1953) points out simply, from the experiment *in vitro* mentioned above, that ascorbic acid not only, as we have seen, stimulates the synthesis of folinic acid, but combats its enzymatic destruction by oxidizing means in aerobiosis. It is because of its antioxidant power that ascorbic acid protects folinic acid.

It remains to be shown more precisely whether the sum of these different processes of economy of folic acid and the citrovorum factor, set in motion by ascorbic acid, makes it possible to realize completely the protective role of the latter against folic-acid deficiency.

2. Inhibition of Destruction of Vitamin B_{12} by Ascorbic Acid

Ascorbic acid makes it possible, as has been previously seen, to ensure, in the absence of vitamin B_{12}, the growth of several microorganisms normally requiring this vitamin. The mechanism of this favourable action remains obscure.

In a dozen of these microorganisms ascorbic acid appears to be effective only in certain limited conditions: the nitrogen source of the medium must be an enzymatic hydrolysate of casein and not a mixture of amino-acids, the activity of ascorbic acid being, moreover, stronger when it is autoclaved with the medium (Welch and Wilson, 1949; Kitay *et al.*, 1950).

An explanation may be found in the fact that, although it is vitamin free, the enzymatic hydrolysate of casein may contain inactive products of oxidation of vitamin B_{12}. In the presence of ascorbic acid, a reducing agent, these products would be reconverted to active vitamin B_{12} (Welch and Wilson, 1949). In fact, there is not really an inhibition of destruction of B_{12} but, on the contrary, a regeneration of this vitamin.

This conception meets serious objections, however: for one thing, several micro-organisms such as *L. acidophilus* 204, *L. acidophilus S*, *L. helveticus* respond to the stimulating influence of ascorbic acid even in the absence of enzymatic hydrolysate of casein; for

concentration in this vitamin of 0 40 μg/g fresh liver in the deficient subjects not receiving ascorbic acid, and 1·36 μg/g in those receiving it. Considering that the sparing effect thus realized is appreciable, the authors suggest the hypothesis of a stimulation by ascorbic acid of the synthesis of thiamine by the intestinal flora. In fact, the economy of thiamine, observed by these authors, is insignificant when considered, not in absolute value, but in relation to the liver concentration in thiamine of a normal subject, which is from 8 to 10 μg/g of fresh tissue.

In conditions nearly similar to those of the previous authors, Terroine, T. (1957*a*) observes, as is shown by Table 5.8, that the addition of 3 per cent ascorbic acid does not improve in any appreciable or systematic manner, the concentration in thiamine of the liver, the muscle or the brain of the deficient rat.

There is no doubt that an examination, not of the average values, but of the extreme ones, does sometimes show that the concentration in thiamine is higher in the group supplemented with ascorbic acid. But, on the one hand, this increase is not at all systematic, and, on the other, it is insignificant in relation to the concentration in thiamine of the control animals.

With doses of ascorbic acid 300 times weaker than the previous ones, Bhattacharya (1957) also does not observe more than a negligible economy of liver thiamine.

However, could not the protective role of ascorbic acid be explained, partly at least, by a delay in the using up of thiamine in the first weeks of the deficiency. In fact, this is not so, for the drop in the liver concentration in this vitamin, during the whole of the deficiency, shows a striking similarity in rate, whether or not the deficient rats are provided with a supplement of ascorbic acid; in both groups the liver concentration in thiamine drops suddenly, in two

TABLE 5.8*

Consequences of the Administration of Ascorbic Acid on the Thiamine Content of Liver, Brain and Muscle of Rats Deficient in Thiamine. Ascorbic Acid is Administered Throughout the Diet Deficient in Thiamine

Nature of diets	Number of animals used	Weight of animals	Liver B_1 μg/g fresh	Muscle B_1 μg/g fresh	Brain B_1 μg/g fresh
		Young rats			
Control diet given *ad lib.*	11	168 (137–214)	12·0 (7·4–15·5)	2·40 (2·0–2·70)	3·80 (3·0–4·80)
Control diet given *in limited quantity*	22	98 (77–123)	10·53 (6·8–16·2)	2·10 (1·40–2·50)	4 0 (3·30–4·50)
Diet deficient in thiamine	22	70 (56–87)	0·61 (0·26–1·40)	0·60 (0·50–0·90)	1·30 (0·80–1·22)
Diet deficient in thiamine+ 3 per cent ascorbic acid	21	144 (114–181)	0·83 (0·19–2·70)	0·60 (0·40–0·90)	1·50 (0·60–2·20)
		Adult rats			
Control diet given *ad lib.*	6	309 (276–341)	7·8 (6·45–11·0)		
Control diet given *in limited quality*	8	227 (118–225)	8·1 (7·2–12·9)		
Diet deficient in thiamine	8	177 (162–212)	0·50 (0·19–0·87)		
Diet deficient in thiamine+ 3 per cent ascorbic acid	11	273 (226–298)	1·10 (0 80–1·52)		

* According to Terroine, T. (1957*a*).

TABLE 5.12*

Consequences of the Administration of Ascorbic Acid on the Pantothenic Acid Content of Rats Deficient in Pantothenic Acid

Nature of diets	Pantothenic acid content in μg/g	
	Liver of pregnant rats	Total body of the young at birth
Diet deficient in pantothenic acid	40·7	6·7
Diet deficient in pantothenic acid +2 g per cent ascorbic acid	50·3	14·2
Diet supplemented with 100 μg pantothenic acid per day	54·6	15·8

* According to Everson *et al.* (1954).

Every possibility of pantothenic-acid deficiency ought then to be entirely ruled out in the presence of ascorbic acid. However, as it has been seen, ascorbic acid does not combat, or does only slightly, the cutaneous symptoms of this deficiency (Daft, 1951; Hundley and Ing, 1953; Terroine, T., 1959*b*) and exercises only a very partial protective action with regard to gestation in pantothenic-acid deficiency (Giroud *et al.*, 1956). Here, there is a troublesome disagreement, necessitating further investigation to determine more precisely the links between the protective power and the sparing power of ascorbic acid in pantothenic deficiency.

3. Vitamin-A Sparing Action by Ascorbic Acid

Ascorbic acid raises the vitamin-A content of young foxes deficient in this vitamin (Basset *et al.*, 1948).

TABLE 5.13*

Consequences of the Administration of Ascorbic Acid on the Vitamin-A Content of Young Foxes Deficient in Vitamin A

Nature of diets	Vitamin-A content	
	of the serum (μg/ml)	of the liver (μg/g)
Diet deficient in vitamin A	0·28	0·15
Diet deficient in A+20 mg ascorbic acid per 500 g of ration	0·63	0·21
Diet supplemented with 50 I U.A. per 500 g of ration	0·40	0·26

* *According to Basset et al. (1948).*

It is possible, though this has not been proved, that the economy in vitamin A, thus achieved, is sufficient to explain, for those who admit this, the protective power of ascorbic acid in vitamin-A deficiency. Indeed, from the above figures, the vitamin-A contents of the animals supplemented with ascorbic acid are about as high as those of the subjects receiving 50 I.U.A./day, a dose which, according to the authors, gives satisfactory growth and general health.

III. FUNCTIONAL SUBSTITUTION BY ASCORBIC ACID FOR ABSENT VITAMINS

It must be stressed that, at present, the functional substitution of ascorbic acid for a given vitamin is not yet founded on any experimental basis. Whatever the conceptions suggested, they are only hypotheses whose experimental confirmation remains to be established.

These hypotheses have been formulated to explain the favourable role of ascorbic acid in thiamine, biotin and vitamin-E deficiencies in higher animals.

A. Functional Substitution by Ascorbic Acid for Thiamine

The functional substitution of ascorbic acid for thiamine is actually difficult to establish. Indeed, if the exact metabolic role of the thiamine is more or less elucidated, that of ascorbic acid remains uncertain; the operations in which it probably intervenes by its redox properties are at present still undefined. It is, therefore, difficult to determine how the functional interrelations work between these two vitamins.

Nevertheless, a suggestion, of a very general order, attempts to explain the intimate mechanism of such a functional substitution. The oxidation cycle of the ternary chains in which thiamine participates is only a long succession of oxidation-reduction reactions. In the absence of thiamine, ascorbic acid might ensure the normal working of these operations by maintaining the required redox potential (Cheldelin and King, 1954).

This hypothesis finds indirect support in the following observation, bringing to light the relation between the substitutional vitamin activity of ascorbic acid and its influence on the level of the redox potential of the environment: the *L. dorner*, which normally requires vitamin B_{12}, can, however, grow in the absence of the latter if its culture medium contains a heavy excess dose of ascorbic acid. In these conditions the level of the redox potential of the culture medium falls from 0·36 to 0·17 volts. The addition of only a weak quantity of ascorbic acid is not enough both to lower the level of the redox potential and to stimulate the growth of the bacillus. It would seem that the ascorbic acid, by decreasing the redox potential level of the culture medium, suppresses the

another thing, the *L. acidophilus* 204 requires, for growth, ascorbic acid and not B_{12} (Kitay *et al.*, 1950). Finally, several examinations *in vitro* show, not a protective action, but a destructive action of ascorbic acid in regard to B_{12} (Gakenheimer and Feller, 1949).

As an hypothesis, would it not be more probable to imagine that below a certain level of redox potential, obtained by the addition of ascorbic acid to the culture medium, the microorganisms acquire the ability to synthetize the vitamin B_{12} and, thus, do without an outside addition of it? However, there would still remain to be explained the case of *L. acidophilus* 204 which, for growth, requires ascorbic acid and not vitamin B_{12} (Kitay *et al.*, 1950).

3. Inhibition of Destruction of Vitamin E by Ascorbic Acid

The importance of the sparing action upon vitamin E achieved by ascorbic acid appears, according to the different cases, very considerable (Dam *et al.*, 1948), moderate (Dam *et al.*, 1951) or nil (Zacharias *et al.*, 1950), as is shown in Table 5.10.

TABLE 5.10

Consequences of the Administration of Ascorbic Acid on the Vitamin-E Content of Chicks Deficient in Vitamin E

Nature of diets	Vitamin-E content of total fat of the chick after 5 weeks of diet (in µg/g)		Vitamin-E content of the plasma of the chick after 5 weeks of diet (in mg per cent)
	Exp. 1	Exp. 2	
Diet deficient in E	15	traces	<0·5
Diet deficient in E +ascorbic acid	110	8	<0·5
Diet supplemented with E	135	31	2
	0·5 g per cent of C 0·01 g per cent of E		50 mg/day/animal of C 3 mg/day/animal of E
References	Dam *et al.* (1948)	Dam *et al.* (1951)	Zacharias *et al.* (1950)

It is very probably by stabilizing the vitamin E still present in the organism becoming deficient and in the deficient diet, that ascorbic acid achieves, to a greater or lesser degree, a vitamin-E sparing action. This stabilization seems to be effected by protecting vitamin E against an oxidizing destruction. This protection is exercised thanks to the reducing power of ascorbic acid (Dam, 1953, 1957). This explanation seems valuable as it applies probably also to the protective effect of ascorbic acid against the destruction of vitamin E *in vitro* (Dam, 1957).

But, can this sparing action fully explain the excellent protective activity of ascorbic acid against exudative diathesis or encephalomalacia of the chick?

C. Vitamin-sparing Action by an as yet Undetermined Mechanism of Action of Ascorbic Acid

1. Riboflavin-sparing Action by Ascorbic Acid

Ascorbic acid does not ensure a systematic and total sparing effect upon riboflavin. As is shown in Table 5.11, the addition of ascorbic acid to the riboflavin-deficient diet of young rats does not determine any riboflavin sparing effect in half the cases; and does ensure, in the other half, an economy of 35 per cent of this vitamin. The mechanism of this inconstant sparing action has not been studied.

TABLE 5.11*

Consequences of the Administration of Ascorbic Acid on the Riboflavin Content of the Liver of Rats Deficient in Riboflavin

Nature of diets	Number of animals used		Weight of the animals	B_2 content of the liver (µg/g fresh)
Control diet given in limited quantity	30		157 (118–216)	19·54 (15·6–23·60)
Diet deficient in B_2	30		125 (78–165)	8·91 (6·6–13·9)
Diet deficient in B_2 +2 per cent ascorbic acid	25	13	165 (123–215)	9·40 (6·9–12·60)
		12	178 (144–230)	12·1 (11·20–15·60)

* According to Terroine, T. (1957*b*).

Thus, it is possible to suggest that the protection exercised by ascorbic acid, in the rat, against the apparent or metabolic symptoms of deficiency in riboflavin, appear to be independent of the realization or the absence of a partial economy of vitamin B_2. The favourable role of ascorbic acid can thus not be explained by a riboflavin sparing action (Terroine, T., 1957*b*).

2. Pantothenic-acid Sparing Action by Ascorbic Acid

It is quantitatively complete, since, as is shown in Table 5.12, the rats deficient in pantothenic acid and supplemented in ascorbic acid have an absolutely normal concentration in pantothenic acid (Everson *et al.*, 1954). The recent results of Terroine, T. (1959*b*) entirely confirm this powerful sparing action of ascorbic acid upon pantothenic acid in deficient rats.

calf? In all the cases when the function of vitamin E seems to be that of an antioxidant, the causes of the inferiority of ascorbic acid as a replacement factor of this vitamin remain therefore to be discovered.

IV. ANTI-INFECTIOUS PROPERTY OF ASCORBIC ACID

The interrelations of ascorbic acid and micro-organisms are of a complex nature since, according to the different cases, ascorbic acid stimulates or inhibits their development.

Sometimes, as has been seen, the growth of numerous lactic bacilli, in particular, is favoured by the presence of ascorbic acid. But often, also, ascorbic acid is no less than a bactericide with regard, for instance, to *Bacillus putressis*, *Proteus vulgaris*, etc. (*c.f.* Bibliography in Eddy and Ingram, 1953). Nevertheless, these anti-infectious properties are at the present moment being debated.

The work of Barboriak and Krehl (1957) suggests that the protective activity of ascorbic acid against pantothenic-acid deficiency may have a bearing on its anti-infectious property. Pantothenic-acid deficiency is, indeed, accompanied by a decrease in resistance to infections, particularly to lung infection.

Now, the authors show that, associated with sulphamides, which probably destroy the intestinal microflora (Wright and Welch, 1944), ascorbic acid loses, totally, its substitutional faculty in the rat deficient in pantothenic acid. It can thus be deduced that ascorbic acid exercises its favourable role through the intermediary of the intestinal microflora, whose composition it reforms.

This conception perhaps find support in the observation of Hundley and Ing (1953), according to which, a precursor of ascorbic acid, glucuronolactone, exercises only a protective action against pantothenic deficiency if it is administered *per os* and not by injection.

It is found, moreover, that the percentage of the serum γ-globulins to which the antibodies belong, rises from 13 to 17 per cent when, from simple pantothenic-acid deficiency, the rat goes through a pantothenic-acid deficiency in the presence of ascorbic acid (Barboriak and Krehl, 1957).

It seems then that, according to Barboriak and Krehl (1957), the causes of the protective role of ascorbic acid against this deficiency may be found in a possible connection between the microbiological conditions of the intestinal tractus, and the processes regulating the synthesis of the blood proteins and specially the γ-globulins.

V. ANTITOXIC PROPERTY OF ASCORBIC ACID

As is recalled by Guggenheim *et al.* (1953), ascorbic acid has the faculty of attenuating or suppressing the toxicity of various substances: potassium cyanide, phenol, lead, anaesthetics, etc.

Thus, it is not surprising that Ekman and Strombeck (1949) make the hypothesis that ascorbic acid could protect the rat against riboflavin deficiency by setting in motion its disintoxicating role. In this way it contributes to the avoidance of the accumulation of products of unfinished reactions because of the riboflavin deficiency. As an example, the authors show that an excess of sulphamides is totally rejected, just as it is in the rat with riboflavin deficiency, while half of the drug administered is eliminated in a non-toxic form in the rat deprived of this vitamin but receiving ascorbic acid. However interesting this may be, this conception needs to be more amply verified.

VI. RELATIVE SPECIFICITY OF THE SUBSTITUTIONAL POWER OF ASCORBIC ACID

Is the remarkable protection exercised by ascorbic acid against various vitamin deficiencies a specific, exclusive, and fundamental faculty of this factor? If it is so, the substitutional power of ascorbic acid would be ineluctably linked with the integrity of constitution of its molecule. Now, this is not so. It is, indeed, found that an isomer of ascorbic acid, *iso*-ascorbic acid, plays the same protective role as ascorbic acid.

normal need of the micro-organism for vitamin B_{12} (Koditschek *et al.*, 1949). The authors do not, however, state whether the presence of ascorbic acid frees the bacillus completely from the need for vitamin B_{12} or whether it confers to it the aptitude to synthetize the vitamin.

It must be noted from the above experiment that only strong doses of ascorbic acid can influence the redox potential of the culture medium. If this observation could be transposed to higher organisms, and if the hypothesis of Cheldelin and King could be confirmed, it could be supposed that only pharmacodynamic doses of ascorbic acid can exercise a substitutional vitamin activity.

The functional substitution of ascorbic acid for thiamine, within the framework of the general redox processes, may be also imagined in the light of the facts reported by Vinas Espin (1952). Ascorbic acid and thiamine are both shown to be powerful phosphorylation agents whose processes condition the reducing power of the tissues. Thiamine deficiency, like that in ascorbic acid, is accompanied by a decrease in the reducing power of the tissues and, simultaneously, by a slowing down of the phosphorylations. Thus, it is conceivable that, in the absence of thiamine, the maintenance of the normal reducing power of the tissues is ensured by ascorbic acid.

It should also be noted that the conception of Cheldelin and King (1954) closely approaches that of Abbati *et al.* (1953), according to whom thiamine deficiency, as also other vitamin deficiencies, upsets the balance between the different factors which intervene in the redox processes. The authors combat this disorder by supplementing, in the rat, the diet deficient in thiamine, not with ascorbic acid but with another fundamental factor, cytochrome c. In the absence of thiamine, the addition of cytochrome c. ensures a normal growth of the animals and suppresses the appearance of several symptoms of deficiency.

B. Functional Substitution by Ascorbic Acid for Biotin

Lichstein (1951) makes the hypothesis that, in the different enzymatic reactions in which it participates, biotin may exercise a unique role as a hydrogen carrier.

To this hypothesis could there not be added a second? Could it not be that ascorbic acid protects the rat so vigorously against biotin deficiency because it replaces the absent vitamin in the hydrogen transfer reactions? But this replacement would be only partial, since, it should be remembered, in the present stage of our knowledge, ascorbic acid can be considered, with certainty, only as a donor, not as a hydrogen carrier. If it were true that ascorbic acid can be substituted for biotin in the redox processes, it would be in those which control the integrity of the skin, fur and gait, since it is particularly this integrity that ascorbic acid contributes in maintaining in biotin deficiency.

But the hypothesis of the execution, by ascorbic acid, of certain functions normally fulfilled by biotin, is far from being entirely satisfactory: it gives no explanation for either the possible attenuation of the preventive power of ascorbic acid during the period of biotin deficiency, or the absence of any curative power of vitamin C against this same deficiency.

It should not be forgotten, moreover, the eventuality of an indirect biotin sparing action by ascorbic acid, an action whose existence and importance remains still to be determined.

C. Functional Substitution by Ascorbic Acid for Vitamin E

It has been seen above that the economy of vitamin E due to the antioxidant power of ascorbic acid was sometimes slight, sometimes even contested. In these conditions, the protection exercised by ascorbic acid against vitamin-E deficiency must result, as is suggested by Zacharias *et al.* (1950), from a substitution of ascorbic acid for vitamin E in the processes normally requiring the presence of the latter.

Since vitamin E and ascorbic acid are both antioxidants, it is probable that ascorbic acid functions instead of and in place of vitamin E as an antioxidant agent (Zacharias *et al.*, 1950). Ascorbic acid could, in particular, be substituted for vitamin E in combating the autooxidation of polyunsaturated fatty acids in all the symptoms of vitamin-E deficiency which, to develop, need in the diet the presence of these acids; these symptoms are, among others, exudative diathesis, encephalomalacia of the chick, depigmentation of the incisors of the rat, and muscular dystrophy. This mode of action of ascorbic acid would be all the more probable in that it has already been previously observed that ascorbic acid, administered at 1 mg/day for two months to rats receiving 18 per cent olive oil, combats the rancidity of the abdominal fats of the animal (Overman, 1942).

However, if there is simply a substitution of one oxidizing agent for another, why cannot ascorbic acid totally replace vitamin E in opposing the symptoms of deficiency enumerated above? Is it because ascorbic acid does not always succeed in penetrating to the level of the tissue affected by the vitamin-E deficiency to protect it? Is it because it does not penetrate in a sufficient quantity? Why can ascorbic acid replace vitamin E in opposing the appearance of muscular dystrophy in the guinea-pig but not in the chick or the

deficiencies is not absolutely specific, nevertheless it is relatively.

But the causes of the remarkable superiority of ascorbic acid over the other redox or antioxidant factors are still unknown.

The position of ascorbic acid in the rH scale (E'o= 0 058)is certainly a factor determining its ubiquitous protective activity; but it is certainly not the exclusive factor. If it were so, thionine, with its potential of E'o=0 062, would exercise a substitutive activity comparable with that of ascorbic acid. Also, Dam *et al.* (1951), on one hand, Moore *et al.* (1953*a*), on the other hand, observe no complete parallelism between the vitamin-E activity of the numerous redox agents previously mentioned, and their respective positions in the rH scale.

The importance of the acid function—conferred by the dienol group of its molecule—appears manifest in certain of the properties of ascorbic acid. Sealock (1942) observes that the excretion of the tyrosyl derivates, in the scorbutic guinea-pig, diminishes not only by the administration of ascorbic acid but, temporarily at least, by that of a whole series of diacides entering into the oxido-reduction cycle of the glucids: fumaric, succinic acids, etc. Even more, a salt generator of acid, such as ammonium chloride, would have the same influence. If the acid function were essential to the substitutional faculty of ascorbic acid in vitamin deficiencies, other acids, or generators of acids, would, by analogy, and to different degrees, exercise this same faculty. Now, succinic acid, like ammonium chloride, has no protective action against thiamine deficiency (Terroine, T., 1957*a*) and citric acid gives no protection against pantothenic-acid deficiency (Barboriak and Krehl, 1957).

An evident superiority of ascorbic acid over the redox agents or the antioxidant agents lies in its absence of toxicity, even in very strong doses.

Other properties could be studied comparatively in ascorbic acid and in other reducing agents: speed and intensity of their intestinal absorption, maintenance or loss of the integrity of their properties after this absorption, degree of penetration to the level of the tissue specifically affected by a given deficiency. These different suggestions have, besides, been made by Dam (1957) and by Evans (1939), in particular to classify the degree of substitutional aptitude of numerous reducing agents in vitamin deficiencies.

VII. CONCLUSION

It is on a confession of ignorance that this general survey finishes, since, as has been said, the causes of the remarkable polyvalent substitutional activity of ascorbic acid against various vitamin deficiencies are as yet unknown. The mechanisms of this activity are also far from elucidated. Also, it is difficult to understand, at the present moment, why the protection exercised by ascorbic acid against a vitamin deficiency is not always definitive. Why does this protection work for one symptom of the deficiency and not for the other? Finally, why does the favourable activity of ascorbic acid vary so widely according to the species of the deficient animal?

In spite of these numerous problems still to be resolved, the vitamin interrelations of ascorbic acid still remain of undeniable interest. This interest is even greater if it is considered that just as ascorbic acid can replace numerous vitamins, these also can, more or less partially, replace ascorbic acid (Terroine, T., 1955).

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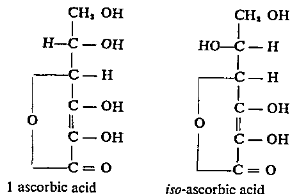
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1 ascorbic acid *iso*-ascorbic acid

In this way, administered at the rate of 2 to 3 per cent in the deficient ration, it ensures as good a growth as ascorbic acid in the rat deprived either of thiamine (Terroine, T., 1957*a*), or of pantothenic acid (Hundley & Ing, 1953). In this latter deficiency it suppresses, quite as well as ascorbic acid, haemorrhage of the adrenals (Hundley and Ing, 1953).

Also, similarly, it stimulates, with the same intensity as ascorbic acid, the growth of the *L. Arabinosus*, placed in a medium low in biotin or pantothenic acid or thiamine (Terroine, T., 1958, Figs. 5.2, 5.5 and 5.6, 1959*a*).

Finally, *iso*-ascorbic acid stimulates quite as *effectively as ascorbic acid, the synthesis of folinic* acid in the rat (Nichol, 1953).

In general, *iso*-ascorbic acid possesses the physico-chemical properties of ascorbic acid (Guggenheim *et al.*, 1953), but, a fundamental difference, it has only one-twentieth of the antiscorbutic power of ascorbic acid (Demole, 1934). So it is not as "vitamin C" that ascorbic acid exercises its protective activity against vitamin deficiencies. Therefore, the relations of ascorbic acid with various vitamins cannot be *sensu stricto* qualified as "vitamin relations".

These facts lead to a halt so far as the understanding of the specific biochemical patrimony of vitamin C is concerned. Indeed, as Cheldelin and King (1954) say, "on one hand, it seems evident that the key property of ascorbic acid is that of a redox agent; on the other hand, scurvy is not cured by non-specific *redox agents, such as iso-ascorbic acid.* Thus, there still remains to be transcribed, in a biochemical equation, the specific participation of vitamin C in one or several enzymatic systems."

Ascorbic acid shares its protective role against vitamin deficiencies, not only with its isomer, *iso*-ascorbic acid, but also, to different degrees, with a whole series of redox agents or antioxidant agents of totally different chemical constitutions.

These factors are either redox dyes, with first methylene blue, then thionine, rosaniline, malachite green, Bindschedler green, methyl violet; or anti-oxidants such as nordehydrogaiacetic acid, antabuse, diphenylparaphenylen diamine; or reducing agents such as cysteine, glutathione, thioglycollic acid.

It is particularly against vitamin-E deficiency that the protective activity of most of these factors has been demonstrated in important works of Dam *et al.* (1953, 1957), of Moore *et al.* (1953*a*, *b*, 1954) and of Draper *et al.* (1958).

Ascorbic acid is also not alone in stimulating the growth of micro-organisms; glutathione or cysteine also ensure a good growth of *L. dorner* deprived of B_{12} (Koditschek *et al.*, 1949) and of *L. arabinosus* in a culture medium low in pantothenic acid (Terroine, T., 1958, Fig. 5, 1959*a*).

The substitutive ability of ascorbic acid in numerous vitamin deficiencies still having, on the whole, some bearing on its fundamental power of participating in redox processes, it is not surprising that a whole series of factors, with the same power, also possess this same substitutive ability.

Ascorbic acid, therefore, seems to exercise its protective property as a non-specific pharmacodynamic redox agent.

It is, then, probably linked, by its redox properties, to various vitamins. That is, reciprocally, these, in spite of their very different characteristics, might *nevertheless have, in common, neighbouring functions* in the redox processes, since they can all be more or less replaced by ascorbic acid.

There remain, however, many obscure points to be resolved. Indeed, although ascorbic acid is not alone in exercising a substitutional activity against vitamin deficiencies, it occupies a privileged position among the other redox agents or antioxidant agents, by the width and extent of its protective properties.

A few examples are sufficient to show this. If methylene blue exercises a substitutional action far superior to that of ascorbic acid in vitamin-E deficiency (works of Dam *et al.*, 1953, 1957), it protects far less well than ascorbic acid against biotin deficiency (Terroine, T., 1954) and it is totally incapable of preventing thiamine deficiency (Terroine, T., 1957*a*). In the same way, although thionine partially combats exudative diathesis of the chick deprived of vitamin E (Dam *et al.*, 1951), it exercises no protective power against thiamine deficiency in the rat (Terroine, T., 1957*a*). Diphenylparaphenylen-diamine prevents the appearance of encephalomalacia of the chick deficient in vitamin E (Draper *et al.*, 1958), but it is powerless to favour the growth and general state in thiamine deficiency in the rat (Terroine, T., unpublished). As against the often important but limited substitutive properties of these factors there is the polyvalent protective power of ascorbic acid. *If, consequently*, the protective role of ascorbic acid against vitamin

deficiencies is not absolutely specific, nevertheless it is relatively.

But the causes of the remarkable superiority of ascorbic acid over the other redox or antioxidant factors are still unknown.

The position of ascorbic acid in the rH scale (E'o= 0 058)is certainly a factor determining its ubiquitous protective activity; but it is certainly not the exclusive factor. If it were so, thionine, with its potential of E'o=0 062, would exercise a substitutive activity comparable with that of ascorbic acid. Also, Dam *et al.* (1951), on one hand, Moore *et al.* (1953*a*), on the other hand, observe no complete parallelism between the vitamin-E activity of the numerous redox agents previously mentioned, and their respective positions in the rH scale.

The importance of the acid function—conferred by the dienol group of its molecule—appears manifest in certain of the properties of ascorbic acid. Sealock (1942) observes that the excretion of the tyrosyl derivates, in the scorbutic guinea-pig, diminishes not only by the administration of ascorbic acid but, temporarily at least, by that of a whole series of diacides entering into the oxido-reduction cycle of the glucids: fumaric, succinic acids, etc. Even more, a salt generator of acid, such as ammonium chloride, would have the same influence. If the acid function were essential to the substitutional faculty of ascorbic acid in vitamin deficiencies, other acids, or generators of acids, would, by analogy, and to different degrees, exercise this same faculty. Now, succinic acid, like ammonium chloride, has no protective action against thiamine deficiency (Terroine, T., 1957*a*) and citric acid gives no protection against pantothenic-acid deficiency (Barboriak and Krehl, 1957).

An evident superiority of ascorbic acid over the redox agents or the antioxidant agents lies in its absence of toxicity, even in very strong doses.

Other properties could be studied comparatively in ascorbic acid and in other reducing agents: speed and intensity of their intestinal absorption, maintenance or loss of the integrity of their properties after this absorption, degree of penetration to the level of the tissue specifically affected by a given deficiency. These different suggestions have, besides, been made by Dam (1957) and by Evans (1939), in particular to classify the degree of substitutional aptitude of numerous reducing agents in vitamin deficiencies.

VII. CONCLUSION

It is on a confession of ignorance that this general survey finishes, since, as has been said, the causes of the remarkable polyvalent substitutional activity of ascorbic acid against various vitamin deficiencies are as yet unknown. The mechanisms of this activity are also far from elucidated. Also, it is difficult to understand, at the present moment, why the protection exercised by ascorbic acid against a vitamin deficiency is not always definitive. Why does this protection work for one symptom of the deficiency and not for the other? Finally, why does the favourable activity of ascorbic acid vary so widely according to the species of the deficient animal?

In spite of these numerous problems still to be resolved, the vitamin interrelations of ascorbic acid still remain of undeniable interest. This interest is even greater if it is considered that just as ascorbic acid can replace numerous vitamins, these also can, more or less partially, replace ascorbic acid (Terroine, T., 1955).

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6

The Role of Carotene and Vitamin A in Animal Feeding

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Contents

I. GENERAL INTRODUCTION

Despite nearly a half century of intense research activity, our knowledge of vitamin A and the related carotenoid pigments is still far from complete. In some fields, studies have proved most rewarding. Chemically, the structures of these substances have been completely established for some years; their synthesis has recently passed from the laboratory to the industrial scale, and low-cost synthetic forms of both β-carotene and vitamin A are now readily available, the latter being already extensively used for animal feeding. Biochemically, investigations of special topics such as the role of vitamin A in the visual process have been spectacularly successful, but the more general systemic function of vitamin A and the carotenoids is hardly understood at all. Our knowledge of the deficiency syndrome is complete only in so far as the partial elucidation of many separate aspects of it. We may catalogue observed symptoms of deficiency, and predict the manner and order in which they will appear in different species, but we lack the biochemical and physiological knowledge to link these together to provide a complete picture of the mode of action of vitamin A under both normal and pathological conditions.

The practical and economic importance of adequate knowledge of vitamin requirements and metabolism is obvious. Recognition of symptoms of vitamin deficiencies, and practical methods for overcoming them in humans and in domestic animals, long precedes our knowledge of the vitamins themselves. More recently, better understanding of the nature of the vitamins and of their utilization by animals, together with improved husbandry practices, has resulted in the virtual disappearance of gross nutritional deficiency symptoms among domestic animals, but there still remain problems in the more nebulous fields of subclinical deficiencies. At the same time, there has been a growing awareness of the producer's responsibility to ensure, through good nutrition of his animals, the optimum nutritive value of the food he produces. Aesthetic considerations have also focused attention on other aspects of vitamin metabolism such as, in the vitamin A field, the colour of animal depot fats, butter and egg yolks. Thus, from the practical point of view alone, we are now no longer interested only in the utilization of vitamin A for the animal's own particular requirements, but in the much wider field of carotenoid pigment metabolism and in the transfer of vitamin A and carotenoids to animal products.

Over the past few years a number of very valuable reviews have appeared dealing with various aspects of carotenoid and vitamin-A metabolism. Much of the material covered relates directly to the role of carotene and vitamin A in animal feeding, and particular reference is made to Deuel (1951, 1955, 1957), Goodwin (1952, 1954), Lowe and Morton (1956), Moore (1953, 1957), Nutrition Society Symposium on vitamin A (1951).

The present review deals mainly with factors influencing the utilization of carotene, particularly from pasture, which is the main source for most domestic animals, with the conversion of carotene to vitamin A, and with the transfer of this vitamin and of the carotenoids to animal products.

In so far as utilization from pasture is concerned, there has been a tendency in the past to assume that herbivorous animals grazing good-quality grass present no vitamin problems, and interest has been limited to their vitamin requirements during stall-feeding periods or under drought conditions. More recent work, particularly relative to the wider interpretation of the problem given above, makes it clear that this is not entirely a safe assumption. A country such as New Zealand, where cattle and sheep are grazed outdoors throughout the year on good-quality pastures, provides excellent opportunity for studies on the utilization of carotenoids from pasture, without the usual complications of winter stall-feeding or summer drought. For this reason, rather, we hope, than parochialism, many of the references quoted relate to New Zealand work.

II. CONVERSION OF CAROTENOIDS TO VITAMIN A

A. The Carotenoid Precursors of Vitamin A

The strongly-coloured carotenoid pigments are entirely of plant origin; the colourless vitamin A is a typically animal degradation product, and all animals are ultimately dependent on plant synthesis of carotenoids for their vitamin A. Only those carotenoids which contain at least one β-ionone ring in their structure are capable of being converted to vitamin A in the animal body, and it is with these that we are primarily concerned. We must, however, join with Goodwin (1952) in deprecating the all too common use of terms such as "biologically active" and "biologically inactive" to distinguish between those carotenoids which can, or cannot, be converted to

β Carotene

Vitamin A

Molecular structures of β-carotene and vitamin A.

all-trans-β-Carotene

3 6-di-cis β Carotene (neo β Carotene B)

α-Carotene

γ-Carotene

Lycopene

Lutein (xanthophyll)

Cryptoxanthin

"Shorthand" structures of various carotenoid pigments showing arrangement of double bonds. Those on left are all -trans isomers. Isomerism may occur about bonds 3, 5, 6, 7 and 9; the structure on the right is a typical neo form.

(* indicates β-ionine rings conferring provitamin A actively on the carotenoid.)

vitamin A. This implies an all too narrow concept of carotenoid function in animals, and their wide distribution, their tendency to accumulate in certain organs and to be selectively mobilized would suggest for these pigments other specific biological functions unassociated with their possible conversion to vitamin A.

From the point of view of human and animal nutrition β-carotene is by far the most important carotenoid. Not only does its concentration in most common feeds greatly exceed that of the other pro-vitamins but, due to its two β-ionone rings, its vitamin-A activity is at least twice as great as those of other pro-vitamins, in which non-vitamin-A-producing ring structures replace one of these β-ionone rings. α-carotene, in amounts up to 30 per cent, and γ-carotene, as traces only, usually accompany β-carotene in many of its plant sources. Cryptoxanthin, another pro-vitamin, occurs in relatively large amounts in yellow maize and is thus important in animal feeding. The nutritionist's interest in other carotenoid pigments, particularly oxygenated derivatives of the lutein type, which are frequently grouped together under the general name of "xanthophylls," is limited mainly to the complications which they introduce into carotene assay procedures. In all, about 80 carotenoid pigments are known to occur naturally, and the chemistry, biochemistry and function of these are fully discussed by Karrer and Jucker (1950) and Goodwin (1952).

In considering pro-vitamin activity of the carotenes, mention must be made of the many spacial possibilities offered by these polyene-type molecules. In general, the most abundant in nature, and the most stable because of their low energy content, are the forms in which all the double bonds exist with trans-configurations. In β-carotene, however, five of the double bonds are not subject to steric hindrance and can rotate to the cis position. These cis isomers, which are described by the prefix *neo* if they are obtained artificially during the processing of the plant material or *pro* if they occur naturally, have vitamin-A potencies considerably inferior to the corresponding all-trans forms. While this decreased pro-vitamin activity must be borne in mind when dealing with dried plant materials, its practical significance in animal nutrition would seem to be fairly limited. At the same time, the possible biochemical significance of cis-trans isomerism in polyene chains, as for example in the visual process (Wald, 1953; Willmer, 1955), should not be lost sight of. Isomerism in the carotenoids has been very fully investigated by Zechmeister and co-workers (*see* review by Zechmeister, 1944).

B. Absorption of the Carotenoids

Factors influencing the absorption of carotenoids have been investigated by a number of workers. Absorption does, however, tend to be defined in different ways, and results must be related to the particular procedure employed. Thus, some workers have measured absorption as the difference between the quantities of carotenoid consumed and that voided in the faeces. This is of very doubtful value since it fails to take into account the destruction of carotenoids in the digestive tract and the possibility of carotene synthesis by intestinal micro-organisms (McGillivray, 1951*a*). Other workers have measured the increased levels of vitamin A attained in the blood or body tissues after or during ingestion of carotenoids. While these appear better techniques, they fail to distinguish between factors which affect absorption of the pro-vitamins and those which influence their conversion to vitamin A. However, this distinction is of academic rather than practical interest, and it may well be that, as pointed out by Moore (1957), adequacy of the enzyme systems involved in the conversion of carotene to vitamin A is the limiting factor in pro-vitamin absorption as compared with the more rapid and complete uptake of vitamin A under comparable conditions.

Despite these difficulties in interpreting experimental results, it seems apparent that most factors described as influencing carotenoid absorption are those which influence, primarily, the absorption of the oily vehicle in which the pigments are usually dissolved. These factors, which have been very fully reviewed by Moore (1957), fall into two somewhat inter-related categories—those concerned with the mode of presentation of the pro-vitamin, and those related to the ability of the species or individual animal to digest the particular food and to absorb fat. These latter considerations open up the very wide fields of digestibility in general, of species differences in fat absorption, and of factors influencing fat absorption within the individual animal, such as the presence of bile, of lipases, etc. The former are more relevant to the present discussion, and the main points may be summarized as follows:

1. The Amount of Carotene Administered

Absorption varies with dosage rate although, as is the general case, digestibility coefficients decrease at higher dosage rates.

2. Presence of Other Substances in the Oily Vehicle

Anti-oxidants such as tocopherol and carotenoids, like xanthophylls and lycopene, which are non-precursors of vitamin A, influence carotene absorption. Although there is some conflict of opinion, most results indicate that these substances present in small amounts enhance carotene uptake, whereas larger amounts are inhibitory, at least to the conversion of carotene to vitamin A. A reasonable explanation for the beneficial action of small amounts of these substances would seem to lie in their protection of the

pro-vitamins against oxidative destruction in the alimentary tract. At higher levels tocopherol interferes, probably again as an anti-oxidant, with the conversion of carotene to vitamin A (see p. 140), and xanthophylls may act likewise through competitive reaction with enzyme systems.

3. Quantity and Quality of the Oily Vehicle

Where carotene is administered in oily solution, increased quantities of fat aid absorption, provided the digestive ability of the particular animal is not exceeded. Absorption is influenced by the triglyceride composition of the fat, but the relationship is not clearly established; poorly absorbed lipoids reduce carotene absorption.

In general, these factors have been investigated at relatively low carotene intake levels. It cannot be assumed that the same effects would be apparent at the consistently higher intakes obtaining, for example, when animals are grazing pasture. Utilization at these higher intake levels has recently been investigated by McGillivray, Thompson and Worker (1958) and by McGillivray and Worker (1958*a*), who found that composition of the vehicle was the most important single factor. Carotene was less efficiently utilized from the hardest and softest fats investigated, and absorption was markedly depressed by the presence of waxes and sterols. At the same time, utilization at these high levels was still influenced by the actual level of carotene intake, and by the concentration of carotene in the oil used as vehicle, but was scarcely affected by the presence, even of large amounts, of tocopherol, xanthophyll or chlorophyll.

The above findings relate only to absorption of pro-vitamins from oil. Carotenoids are only very slightly soluble in vegetable oils, and in many plant sources the concentration of carotene is much greater than their fat could be expected to dissolve. In these cases the pigment must exist in forms other than oily solution. Strauss (1942) reported a carotene–protein complex in the juice of carrots, and, more recently, Nishimura and Takamatsu (1957) have isolated a similar complex from parsley leaves. The same situation probably obtains in pasture grasses and clovers where the triglyceride fat content is extremely low, hence a fair proportion of the carotene, and the even more abundant xanthophyll, must exist in non-oily solution.

No information is available regarding the utilization of these carotene–protein complexes by animals. So-called aqueous dispersions of carotene, prepared by using surface-active agents of the Tween type, have been investigated by several workers. Burns, Hauge and Quackenbush (1951*a*), and Kon, McGillivray and Thompson (1955), found evidence of more rapid and greater carotene absorption from aqueous dispersions than from oily solution, although in calves, the actual amounts of vitamin A stored in the livers were the same, despite the more rapid appearance and higher concentrations of carotene in the blood following dosing with aqueous dispersions. These dispersions are not, however, strictly comparable to the carotene–protein complexes. Dispersing agents like the Tweens could presumably be absorbed unchanged, whereas the protein materials may be subject to breakdown by the digestive proteases, leaving the carotene in a virtually non-digestible form. Clearly further work is required, and a more thorough investigation of the mode of occurrence of carotenoids in plants and of the absorption of the different forms, may provide an explanation for the relatively inefficient utilization of pro-vitamins from leafy plant material.

C. Special Factors Influencing the Absorption of Carotenoids by Ruminants

1. Rumen Activity

Ruminants are notoriously inefficient in their utilization of carotene from pasture. Although no direct evidence is available, it is interesting to speculate on the possible effect of rumen activity on subsequent carotenoid absorption. Of the various changes taking place in the rumen, hydrogenation of lipid material might be expected to have the greatest effect on the subsequent absorption of the carotenoids. Pasture fats are highly unsaturated (Shorland, 1944) but recent work has shown that a considerable amount of hydrogenation may occur during passage through the rumen (Shorland, Weenink and Johns, 1955). This modification of pasture fats may have a profound influence on carotene absorption since, as already mentioned, the degree of unsaturation of the oily vehicle has a marked effect on the absorption of carotene at high intake levels (McGillivray *et al.*, 1958). Furthermore, although this aspect does not appear to have been investigated, it seems probable that carotenoids themselves might be susceptible to hydrogenation in the rumen, with resultant loss of pro-vitamin activity.

2. Bloat Prophylaxis

Bloat is a serious problem with ruminant animals. Measures recently introduced in New Zealand for its control may cause significant decreases in carotenoid absorption. Reid and Johns (1957) have found that the most reliable materials are fats and oils, which act as anti-foaming agents, and current practice is to drench the animals, or spray the pastures, with suitable oils. Animal and vegetable oils have little or no effect on carotene absorption (McDowall, Patchell and Reid, 1957), but mineral oils of the heavy liquid

paraffin type, which are equally effective and considerably cheaper, are known to reduce carotene uptake. Thus, even the small amounts of mineral oil necessary to prevent dustiness in mineral mixtures and lucerne leaf meal (0 08 per cent in the diet) were found to have a deleterious effect on carotene utilization (Burns, Hauge and Quackenbush, 1951*b*). The dosage rate recommended for bloat control (about 150 ml per cow per day) is considerably in excess of this and has been found to reduce carotene absorption by 40-50 per cent (McDowall, McGillivray and Reid, 1957). There is a corresponding decrease in intestinal vitamin-A formation, and these effects of paraffin ingestion are reflected in marked reductions in the carotene and vitamin-A ester levels in the blood and milk fat. The implications of this reduced vitamin uptake, both from the point of view of the well-being of the animal itself, and the nutritive value of the dairy products, are obvious. However, the incidence of bloat in a herd is usually limited to a period of about six weeks in the early spring. At this time of the year carotene absorption from pasture is normally high (*see* p. 143). The animals appear to suffer no ill-effects from the reduced fat-soluble vitamin intake, and it has been concluded that the economic advantages in the use of heavy liquid paraffin over this short period, and at dosage rates not exceeding those given above, outweigh the nutritional disadvantages resulting from its use (McGillivray, McDowall and Reid, 1959).

D. Site of Conversion

When Moore (1930, 1931) first demonstrated the formation of vitamin A from carotene, in the rat, it seemed unnecessary to look beyond the liver as the site of conversion. Carotene persisted, apparently unchanged, throughout the intestinal tract, so that conversion appeared to be an extra-intestinal function; the liver, with its intense biological activity, was the most likely organ, and it was found to contain small amounts of unchanged carotene as well as large stores of vitamin A. Support for this suggestion was provided by early experiments by Olcott and McCann (1931), in which they incubated livers from vitamin-A-deficient rats with carotene and claimed the formation of vitamin A through the agency of an enzyme system which they referred to as liver "carotenase." Subsequent attempts to confirm and extend these findings led to equivocal results, but for over twenty years it was still generally accepted that in the intact animal conversion took place in the liver.

However, it became increasingly difficult to reconcile the concept of hepatic conversion with other findings regarding the utilization of carotene and vitamin A, and long before the first direct evidence was produced in support of an alternative site of conversion there were growing doubts about the part played by the liver in the conversion of carotene to vitamin A. When convincing evidence was produced against the hepatic theory of conversion, it is not surprising therefore that it should come almost simultaneously from several laboratories. Foremost in the field were groups headed by Deuel at California, Kon at Reading and Morton at Liverpool, and as a result of this work it is now clearly established that, in the wide range of species investigated, the main site of conversion is the wall of the small intestine. Evidence in support of this has been fully reviewed by Kon and Thompson (1951), to whom is due the credit for the most complete and convincing experiments on intestinal conversion (Thompson, Ganguly and Kon, 1949*a*; Thompson *et al.*, 1950), and may be briefly summarized as follows:

(*a*) Oily solutions or colloidal preparations of carotene injected parenterally are not converted to vitamin A, the carotene merely accumulating unchanged in the liver and other tissues.

(*b*) The blood of many species is virtually free of carotenoids; in these cases oral dosing with carotene produces little or no increase in the amount of pigment present in the portal blood or intestinal lymph, so that there is no mechanism for the transport of appreciable amounts of carotene to the liver.

(*c*) Following oral dosing with carotene, vitamin A appears in the wall of the small intestine before it can be detected in the liver or other tissues; subsequently, vitamin A appears in the intestinal lymph, in the systemic blood and, finally, in the liver; tapping and removing the intestinal lymph by cannulation prevents this appearance of vitamin A in the blood and liver.

Obvious extensions of this work were attempts to demonstrate conversion *in vitro*. Early in this field were Wiese, Mehl and Deuel (1947), who claimed *in vitro* conversion in rat intestines incubated with carotene. These findings have been confirmed in the rat by Rosenberg and Sobel (1953*a*, *b*) and extended to calf (Stallcup and Herman, 1950) and sheep (McGillivray, 1951*b*) intestines. In contrast, however, Glover, Goodwin and Morton (1948) and De and Sundararajan (1951) in the rat, and Bieri and Pollard (1953) in the rat, rabbit and calf, were unable to demonstrate any increase in vitamin A after incubating whole intestines with carotene. Negative results were also reported by Kon and Thompson (1951) in the rat, using the perfusion technique of Fisher and Parsons (1949), and, more recently, a critical re-examination of most of the methods employed (Worker, 1959) has failed to substantiate claims to have effected conversion *in vitro*.

These negative findings, however, in no way detract from the unequivocal evidence in support of the intestine as the main site of conversion of carotene to vitamin A, in the intact animal. They merely illustrate some of the difficulties inherent in this type of work, where negative findings may be ascribed to faulty technique, and positive results to a failure to distinguish between true vitamin A and the complex mixture of oxidation and isomerization products likely to be formed from the carotene molecule.

E. *Alternative Sites of Conversion*

As already mentioned, the blood of many species is virtually free of carotene, but in others, particularly cattle, relatively high concentrations are present in the blood and tissues. With the wall of the intestine established as the main site of conversion of carotene to vitamin A, the question naturally arises as to whether, in these latter species at least, there may not be a secondary site, or sites, where vitamin A may be formed from the relatively large amounts of carotene which escape conversion during passage across the intestinal wall. There is no direct evidence on this point; what information we do have is based on experiments with parenterally administered aqueous dispersions of carotene solubilized with surface-active agents of the Tween type. The extent to which we may argue from the fate of these highly unphysiological preparations is clearly limited but, at the same time, differences in their utilization may provide clues to the significance of blood and tissue carotenoid levels in various species.

As already mentioned, oily solutions or colloidal preparations of carotene injected parenterally are not converted to vitamin A. Indeed, investigations in this field led to the first suggestion, by Sexton, Mehl and Deuel (1946), of intestinal conversion of carotene to vitamin A. An observation in the same year (Tomarelli, Charney and Bernhart, 1946) that carotene was slightly more effective in promoting growth when injected as an aqueous dispersion in Tween 80 than when administered orally, would seem to have been overlooked in subsequent work on the site of conversion. Perhaps this is just as well since, at that time, this finding would have lent considerable weight to the hepatic theory. More recently, however, a number of workers have confirmed the findings of Tomarelli *et al.* (1946). Bieri (1949), Bieri and Schultze (1951), Bieri and Sandman (1951) and Bieri and Pollard (1954) working with rats; Hentges, Grummer and Sorensen (1952) with pigs; Church *et al.* (1954) with sheep; Kon *et al.* (1955) with rats and rabbits; Bieri (1955) with chicks and rabbits; and Worker (1959) with rats, guinea-pigs and sheep, have all shown conclusively that, in these species, parenterally-administered carotene, provided it is presented in a suitable physical state, may be converted to vitamin A. In contrast Church *et al.* (1954) and Kon. *et al.* (1955) found no indication of conversion under similar conditions in calves. It is thus significant that the ability to utilize parenterally-administered aqueous dispersions of carotene appears to be restricted to those species which do not normally circulate carotenoids in their blood. Some difference in the catabolic pathway of carotene in species like the bovine would seem to be the likely explanation, and this is borne out by observations on the rates at which intravenously-administered aqueous dispersions of carotene disappeared from the blood of different species (Kon *et al.*, 1955). In non-carotenoid accumulators, this carotene disappears from the blood in 24 hours, whereas, in calves, relatively high levels persist for a considerable period after injection. In the same way, under normal conditions of oral administration, large quantities of carotene may appear in the blood of the bovine merely because an efficient mechanism for its destruction is lacking.

In species other than the bovine, it is the general conclusion of the workers cited above, and of subsequent investigations (McGillivray, Thompson and Worker, 1956; McGillivray and Worker, 1957 and 1958*b*; Worker, 1957), that the ability to convert carotene to vitamin A is not limited to the wall of the intestine but is common to many, and perhaps to all, tissues. Under normal circumstances, however, the practical significance of these other sites of conversion is limited by the relative impermeability of the intestinal wall to unchanged carotene. At the same time, some species such as sheep, which are generally regarded as non-accumulators of carotenoids, do absorb small quantities of carotene. Extra-intestinal sites of conversion may prevent this carotene accumulating in the blood and tissues, and may contribute to the animal's requirements of vitamin A.

It would appear that the mechanism of conversion is similar, whether in the wall of the intestine following oral dosing of carotene, or in other tissues following parenteral administration of aqueous dispersions. If this is so, the latter technique affords a convenient method for studying the factors which influence the actual conversion mechanism, as distinct from those which exert only an indirect effect through their control over intestinal absorption of the oily vehicle or stability of carotene through the digestive tract. Thus, it is well established that thyroid status has a marked effect on the utilization of orally-administered carotene (*e.g.* Cama and Goodwin, 1949). McGillivray *et al.* (1956) and Worker (1956) have shown that the conversion of intravenously-administered carotene is unaffected by hyper- or hypo-thyroid conditions, and

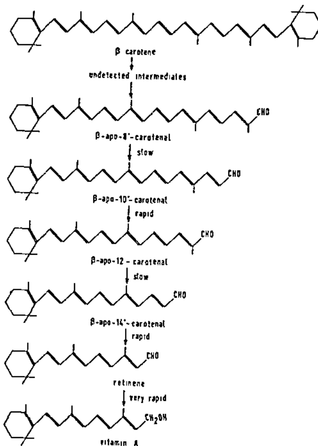
conclude that with oral administration, thyroid status is important only in so far as it affects fat absorption. In contrast, tocopherol has been shown to have a direct effect on the conversion mechanism, increased amounts of tocopherol markedly inhibiting the formation of vitamin A from intravenously-administered carotene (McGillivray and Worker, 1958*b*). Further studies in this field should prove most rewarding.

F. The Conversion Mechanism

The mechanism by which carotene is converted to vitamin A must be regarded as one of the major unsolved problems in the vitamin-A field. Consideration of the formulae of the two molecules, β-carotene and vitamin A, has led many workers to regard the conversion reaction as a hydrolytic fission at the central double bond of the β-carotene molecule to yield two molecules of vitamin A. Attempts to carry out this rather unusual reaction chemically have failed, and Hunter (1946) has suggested, as an alternative mechanism, oxidative attack at the central double bond of β-carotene to yield two molecules of vitamin A aldehyde, which are then reduced to vitamin A. Chemically, β-carotene can be oxidized by hydrogen peroxide to give a good yield of aldehyde (Hunter and Williams, 1945; Wendler, Rosenblum and Tishler, 1950) but, in keeping with physico-chemical theory, Glover and Redfearn (1954) have shown that the initial attack is at a terminal, rather than the central, double bond. A somewhat similar attack *in vivo* would produce a series of aldehydic intermediates of decreasing chain length. Glover and Redfearn prepared a number of these possible intermediates and showed that they could all be converted to vitamin A, in the rat. Thus, they suggest a type of β-oxidation of the carotene molecule to form vitamin A aldehyde:

It might be anticipated that the oxidation could proceed beyond the vitamin-A aldehyde stage but, up to that point, the methyl groups are in the α-position to the carbonyl group, whereas vitamin-A aldehyde is substituted in the β-position. Branched chain fatty acids are oxidized *in vivo* when the methyl group is in the α-position, but not when it is in the β-position, to the carboxyl group (Carter *et al.*, 1939). Whether this observation can be extended to polyene chains of the carotenoid type is open to question, since Redfearn (1957) has shown that β-methyl groups in higher homologues of vitamin A do not appear to inhibit further oxidation. However, vitamin-A storage from this type of compound was much less than from the corresponding compound with an α-methyl group, so that, if the β-methyl group does not completely inhibit oxidation, at least it may retard it sufficiently to allow, in the case of a compound like vitamin-A aldehyde, some vitamin-A formation. Furthermore, substances which have identical spectroscopic and chromatographic properties to possible aldehydic intermediates between β-carotene and vitamin A have been obtained from the intestine during carotenoid absorption (Festenstein, 1951, as quoted by Glover and Redfearn).

It would seem probable, therefore, that provitamins are first transformed into vitamin-A aldehyde by stepwise oxidation from one end of the molecule, and then reduced to vitamin A. This mechanism, involving the formation of one molecule of vitamin A from one molecule of β-carotene, is in accord with the generally accepted biological activities for the two substances. (The new International Standard for vitamin A, established after a long series of collaborative trials, is based on 50 per cent conversion of carotene to vitamin A, *i.e.* 0 6 μg β-carotene is biologically equivalent to only 0·3 μg vitamin-A alcohol (Hume, 1951)).



Possible mechanism, as proposed by Glover and Redfearn (1956), for the conversion of β-carotene to vitamin A by a process of β-oxidation (after (*Lowe and Morton*, 1956).

It is, however, in conflict with the equivalent biological potencies, reported by Koehn (1948) and Burns *et al.* (1951*a*), for β-carotene and vitamin A at low levels of intake in the presence of adequate tocopherol, and it is difficult to explain these findings in relation to the weight of evidence in support of oxidative degradation of the carotene molecule.

An attractive feature of Glover and Redfearn's scheme is that it involves only the normal β-oxidation processes and does not introduce specific enzymes such as "carotenase." Thus, the ability to convert carotene to vitamin A would not be restricted to the intestinal wall, but would be common to all cells whose mitochondria contain the enzyme systems necessary for the degradation of fatty acids. This is in line with the work already discussed on extra-intestinal conversion, but it is difficult to explain the clearly-defined species differences in carotenoid metabolism, particularly following parenteral administration, if β-oxidation only is involved. Attention might, however, be directed towards differences in the physical state of the substrate, even immediately following intravenous ingestion of carotene dispersions, rather than to possible deficiencies in the enzyme systems themselves. Even in species which can convert parenterally administered aqueous dispersions of carotene to vitamin A, there is evidence of a rapid change in the physical state of the dispersed carotene in the blood (McGillivray and Worker, 1958*b*) and this, coupled with species differences in the specific plasma proteins available for carotenoid transport (Ganguly *et al.*, 1952), could markedly influence subsequent carotenoid metabolism, irrespective of the particular enzymes present.

III. SPECIES DIFFERENCES IN CAROTENOID METABOLISM

As already discussed, all higher forms of animal life have the unique ability to convert dietary carotene to vitamin A. However, apart from this common feature of carotenoid metabolism, there are marked species, and sometimes variety differences in the way in which animals absorb, reject or modify carotenoid pigments. Domestic mammals fall into two categories—those which do not accumulate carotenoids in their tissues, and those which accumulate mainly carotenes. Sheep and pigs are typical of the former group, and cattle and horses of the latter group. Birds, on the other hand, are primarily xanthophyll accumulators, as also are fish. A third group of mammals, of which the human is the best example, non-selectively absorb, and deposit in their tissues, all carotenoids fed, but domestic animals are not represented in this group.

Mammals which do not accumulate carotenoids in their tissues are more common than those of the other groups (*e.g.* survey by Jensen and With, 1939). Whether this failure to accumulate carotenoids is due to less efficient absorption of the pigments or to their more rapid and complete breakdown in the wall of the intestine is not known. In addition to the enzyme systems involved in the conversion of carotenes to vitamin A, it is probably necessary to postulate the presence of systems capable of converting non-provitamin carotenoids to colourless and, as yet, unidentified products. In the absence of more precise knowledge of the catabolic pathways involved, no definite theories can be advanced, but, as discussed by Goodwin (1954) and Deuel (1957), differences in the efficiencies of these systems would explain observed differences in carotenoid metabolism.

As already mentioned, it is of interest to note that the intestinal wall is not a complete barrier to carotenoids, even in typical non-accumulators, and various workers have shown that under conditions of high carotenoid intake, small amounts of carotene can be detected in the blood and lymph. Thus, Thompson *et al.* (1950) found low concentrations of a pigment, which they considered to be β-carotene, in the intestinal lymph of a pig dosed with carotene in oil, and readily detectable quantities of carotene have been reported in the blood of sheep on green feed (Peirce, 1945; Paulsen, Hilmoe and Moxon, 1944).

However, although they indicate some carotenoid absorption, these traces of carotene are insignificant when compared with the levels normally found in the blood of cattle or even of horses.

A. Significance of Blood Carotenoid Levels

It is difficult to assess the significance of the high levels of carotenoids which may be reached in the blood and tissues of cattle. There have been many reports of widely varying specific effects of carotenoids, as such, in the animal body (*e.g.* review by Goodwin, 1952) but none of these appears to have been unequivocally confirmed. In as much as some of these claims have been based on experiments with tissues which, in the species studied, do not normally contain carotenoids, their significance in the intact animal may be very limited.

There is no clear evidence of extra-intestinal conversion of blood or tissue carotene to vitamin A, but the problem is by no means fully investigated. In any event, whether vitamin A is a product or not, a mechanism exists for the very rapid destruction of carotenoids in the bovine. Based on the rate of change

in blood-content of carotene associated with changes in carotene intake, McGillivray (1957*a*) calculated a daily breakdown of 40-50 mg carotene in pasture fed cows. Although this figure seems high, it represents only about 1 per cent of the carotene present in the feed. It is about the same as the amount of β-carotene which would need to be broken down in the wall of the intestine to yield the 25 mg vitamin A which he also calculated was formed each day. The remaining 98 per cent of the ingested carotene is either destroyed in the lumen of the intestine or voided in the faeces.

Although their physiological significance is probably very limited, blood carotene levels are of considerable nutritional interest. Their value here lies in the way in which they may be used to follow carotene absorption and conversion to vitamin A. Within any one breed, and provided the diet contains no preformed vitamin A, there is a general relationship between the levels of carotene and of the esterified, transport form of vitamin A in the blood. These vitamin-A ester levels, in the blood of pasture-fed animals, for example, are too low for small changes to be measured with any great degree of accuracy unless inconveniently large quantities of blood plasma are extracted. Blood carotene levels, on the other hand, are high and easily measured, they respond rapidly to changing carotene utilization, and are a good index, not only of carotene absorption, but also of conversion to vitamin A.

B. Blood Carotenoid Levels in the Bovine

A far higher concentration of carotenoids appears in the blood and body tissues of the bovine than of any other species. It is convenient therefore to limit consideration of factors influencing blood carotenoid levels to the bovine, in which they have been more extensively studied than in any other species. It is probable that the same factors, modified by the species differences in carotenoid metabolism already referred to, will operate in other species as well.

1. FEED

It is well recognized that, as might be expected, the carotenoid content of the feed is the main factor affecting the level of these pigments in the blood. This is particularly apparent where cows are transferred from low-carotenoid winter rations to spring pasture, Lord (1945), for example, quoting average carotene figures from as low as 130 μg/100 ml plasma during the stall-feeding period to as high as 1680μg/100 ml for cows on pasture.

There are, thus, clearly-defined seasonal trends, in most countries, in the carotenoid content of cows' blood associated with periods of stall and pasture feeding. A somewhat different situation exists in countries such as New Zealand, where cows are outdoors on pasture throughout the year. Here again, despite the relatively uniform carotenoid intake, marked seasonal variations are apparent in the blood carotene and xanthophyll levels. McGillivray (1957*b*) found that blood carotene increased steadily during early spring (immediately after calving) to an average value of approximately 1700 μg/100 ml plasma in late spring (October–November). Thereafter, there was a steady decline to levels in the order of 800 μg/100 ml in mid-summer (January–February), followed by increases during the autumn. Xanthophyll levels showed similar trends, ranging from an average of 110 μg/100 ml in spring to 40 μg/100 ml in mid-summer. More recent work, extending over several seasons (McGillivray, 1959), has confirmed these carotenoid trends, although the average values in typical New Zealand herds throughout the season would appear to be about 35 per cent higher than those quoted above. These figures again illustrate the striking difference in carotene metabolism between cattle and sheep, since the blood of sheep grazing similar pastures contained only 1 to 2 μg carotene per 100 ml.

They also illustrate the selectivity of carotenoid absorption in the bovine (or differences in the rates of metabolism of carotenoids) since, although pasture contains twice as much xanthophyll as carotene, the concentration of carotene in the blood is about 15–20 times that of xanthophyll.

The daily carotene intake of these cows was approximately 5 g. It varied somewhat from day to day, depending on the type of pasture, but remained relatively constant throughout the season, the management of the pastures normally ensuring lush feed even in mid-summer. Clearly, therefore, factors other than the carotene content of the feed influence the levels of carotenoids in the blood of cattle.

McGillivray also found a similar seasonal trend in blood vitamin-A ester levels, indicating that the reduced absorption over the summer months was influencing the amount of carotene converted to vitamin A. A striking reflection of these varying blood vitamin A and carotenoid levels is the seasonal trend in the vitamin-A potency of the milk fat of pasture-fed cows (*see* p. 148).

Other workers (*e.g.* Sutton and Soldner, 1945) have found evidence of similar seasonal trends in the blood of pasture-fed cows and have ascribed them to a drying up of the pasture with, presumably, reduced carotene intake. As is discussed later, in relation to the vitamin-A potency of milk fat (p. 147), it does not appear that the same explanation applies under New Zealand conditions.

2. Breed

It is well recognized that breed has a marked effect on the carotenoid content of the blood of cows. Of the more common breeds, Guernseys appear to have the highest levels of carotene in their blood. On the same feed, Jerseys reach about 60 per cent of the Guernsey figure, and Holsteins and Ayrshires about 50 and 45 per cent respectively (e.g. Sutton, Kaeser and Soldner, 1945; Wise *et al.*, 1947).

3. Individuality

Although, in general, the breed differences referred to above are clearly defined, it is of interest to note that just as great variability may be shown between animals of the same breed kept under the same conditions. Thus, the level of carotene in the blood of individual pasture-fed Jersey cows has been found to range from half to over double the herd average, some animals reaching and maintaining levels in excess of 5,000 μg/100 ml plasma (McGillivray, 1960).

Individual animals also show marked differences, not only in the absolute levels of xanthophylls in their blood, but also in the ratios of carotene to xanthophylls. In contrast, monozygous twins are extremely uniform in their blood carotenoid pictures, and the whole question of animal variability in blood constituents should form the basis of a most interesting genetic study.

4. Sex

A number of workers have found that the carotenoid level of cow blood plasma is up to five times greater than bull plasma (*e.g.* Gillam and El Ridi, 1937, 1945; Sutton and Soldner, 1945). Although sex differences are common in carotenoid and vitamin-A metabolism, it seems that the explanation here lies in different management, which frequently restricts the access of bulls to grass to very short periods. Where bulls are run on pasture throughout the year their blood plasma carotenoid content is similar to that of cows of the same breed and under the same management (McGillivray, 1960).

5. Lactation

Marked decreases in the blood carotenoid content of cows near parturition have been reported by a number of workers (*e.g.* Walker *et al.*, 1949). Although formerly ascribed to hormonal imbalances associated with parturition (Goodwin and Wilson, 1951), more recent work (Thompson and McGillivray, 1957; McGillivray, 1959) has shown that these decreases result directly from the abnormally high uptake of carotenoids by the mammary gland for colostrum formation. Once lactation is established it would appear to have no further effect on blood carotenoid levels, which were found to be almost identical, throughout the season, in a group of lactating cows and in their non-lactating monozygous twin mates (McGillivray, 1960).

6. Age

At birth, the level of carotene in the blood of the calf is extremely low. Moore and Berry (1944) quote, for calves of four different breeds, average figures of 2·4 μg/100 ml plasma at birth, rising to 25 μg on the first day following suckling. In an experiment, in which Holstein calves were fed on milk and were denied access to green feed for up to three months, the present author (unpublished results) found that blood carotene levels remained consistently low (average 4·5 μg/100 ml plasma), whereas comparable animals, with access to pasture, had blood carotene levels in the order of 300–400 μg/100 ml.

Once the animals are completely on pasture, blood carotenoid levels increase with age, the blood carotenoid content of year-old pasture-fed heifers, for example, being about 74 per cent of that of comparable adult cows. Even in their first season of lactation young cows are still slightly lower than more mature adults, but thereafter there appears to be little change either in carotene or in xanthophyll levels (McGillivray, 1960).

IV. TRANSPORT, STORAGE AND MOBILIZATION OF VITAMIN A

Whether vitamin A is absorbed as such, or is formed there from carotenoids, it is transported from the intestine, via the lymphatic system, to the systemic blood, and thence to the liver, the main storage organ (Eden and Sellers, 1949; Thompson *et al.*, 1949*a*, 1950). During absorption, esters of vitamin A are at least partially hydrolysed, but re-esterification occurs in the wall of the intestine, since the vitamin A appearing in the lymph is predominantly in the esterified form irrespective of whether it was ingested as alcohol, ester or pro-vitamin. The actual form in which these esters are transported has not been investigated, but it seems reasonable to assume that they are associated with fat of immediate dietary

origin and, in the case of carotenoid accumulators, with varying amounts of freshly absorbed pigment, in the chylomicrons.

This material, discharging into the systemic blood, causes a rapid increase in the vitamin-A ester content of the blood. Since, in the blood of fasting animals, the vitamin A, maintained from liver reserves, is almost completely in the free alcohol form (Thompson *et al.*, 1949*a*), a fractionation of blood vitamin A into alcohol and ester forms, using techniques such as that described by Thompson *et al.*, provides a very complete picture of the vitamin status of the animal. It must be noted, however, particularly during the absorption of large amounts of vitamin A, that the increases in blood ester content may be accompanied by parallel but relatively smaller, and equally transitory, alcohol increases. (*See*, for example, blood changes in the bovine following large oral doses of vitamin A (McGillivray, 1957*c*)).

Vitamin-A ester is fairly rapidly removed from the blood and stored in the liver, where extremely high concentrations may accumulate, depending on the species and the level of vitamin in the diet. In the liver, the vitamin is again mainly in the ester form, probably as the palmitate (Gray and Cawley, 1942). A portion, variable in amount but frequently in the order of 10 per cent of the total, is present in the free alcohol form. There is some evidence that these two forms differ in their distribution in liver cells, and Glover, Goodwin and Morton (1947) have suggested that the free alcohol present in the liver cells is probably associated with protein, whereas the ester form is in the Kupffer cells. Blood vitamin-A alcohol levels may be related to the quantities of vitamin-A alcohol in the liver (Glover *et al.*, 1947) but are certainly independent of total hepatic stores, except in animals approaching a state of vitamin-A deficiency, where there is a relationship in the final decline of blood and liver contents.

Under normal conditions, blood vitamin-A alcohol is maintained from liver reserves at a remarkably constant level, usually for all mammals in the range 20–35 μg/100 ml plasma. In the blood, the vitamin exists in combination with plasma proteins, although evidence is incomplete regarding the particular protein or proteins involved. Specific plasma proteins available for carotenoid and vitamin-A transport may vary with species (Ganguly *et al.*, 1952).

The level of vitamin-A alcohol in the blood must be regarded as an equilibrium between rates of removal by the tissues and of replacement from liver stores. Factors known to influence blood vitamin-A alcohol levels (*see*, for example, review by Moore, 1957) may, therefore, affect release from the liver, or uptake by the tissues, or both. In some instances, such as the effect of fever or of cortisone treatment, we may clearly postulate one or other action, but the process of mobilization of liver reserves of vitamin A must be regarded as a complex series of reactions involving, possibly, initial hydrolysis of the palmitate, coupling of the alcohol with liver proteins, transfer to a plasma protein complex and, finally, uptake of this, either intact or after breakdown, by the tissues. Furthermore, as yet we know nothing of the ultimate fate of the vitamin A after it is released from the liver, since it cannot be traced into other tissues or into body secretions in unchanged forms. This whole field is an intriguing one, but progress in it must await a better understanding of other physiological changes associated with factors, particularly the hormones, already known to influence blood levels of vitamin-A alcohol.

It is clear, however, that the liver does represent a reserve store on which the animal may draw during periods of vitamin-A deprivation. Under these conditions the time of survival is roughly proportional to the magnitude of the liver storage. The release of vitamin A from the liver is not always sufficiently rapid to meet particular stresses. Thus, the abnormally large demands of the mammary gland for colostral formation cause a transitory decline in blood vitamin-A alcohol levels near parturition, similar to that already noted for carotenoids (Walker *et al.*, 1949).

At the same time, the liver cannot be regarded as a passive store of the vitamin. While we may assume that body requirements will remain relatively constant and independent of liver reserves, vitamin A would appear to be lost from the liver at a rate roughly proportional to the magnitude of the reserve remaining at that time (Davies and Moore, 1935, 1937; Frey and Jensen, 1947). In other words, high liver reserves are not employed to the best advantage, the vitamin being either destroyed in the liver or released at an unnecessarily rapid rate for destruction elsewhere in the body. In this connexion Gerber, Raab and Sobel (1954) quote a most interesting case of a woman suffering from extreme hypervitaminosis A through prolonged ingestion of massive doses of vitamin A. During the two and a half months she was under observation following the cessation of dosing, the vitamin-A content of her blood remained abnormally high, the excess vitamin A being mainly in the esterified form. To our knowledge this is the only report of measurable quantities of vitamin-A ester, of other than immediate dietary origin, appearing in the blood. The condition may of course be peculiar to acute hypervitaminosis A but, at the same time, a release of vitamin A from the liver as ester, rather than as alcohol, may represent an alternative catabolic pathway for the expenditure of high liver reserves. Under these conditions the actual level of ester in the blood could remain too low to

detect easily, but might still represent a marked drain on liver reserves.

The rapid expenditure of high liver reserves emphasizes the desirability of small regular intakes of vitamin A rather than isolated massive doses. However, the economics of the problem may favour the latter, though wasteful, procedure. Thus, the massive dose technique has been successfully employed with sheep under drought conditions in Australia where more regular dosing would be quite impracticable (*e.g.* Franklin *et al.*, 1955).

V. TRANSFER OF CAROTENOIDS AND VITAMIN A TO ANIMAL PRODUCTS

A. General

The transference of vitamin A and its pro-vitamins to animal products such as milk and eggs is of importance from the points of view of human nutrition and of meeting the initial vitamin requirements of the young animal.

1. Human Nutrition

The contribution of milk and milk products to human vitamin-A requirements is quite considerable. This follows, not only from the actual vitamin content of milk fat, which under the best farming and processing conditions is relatively high, but also from the facts that the fat provides an efficient vehicle for absorption, and that over half the activity is derived from preformed vitamin A, which is well known to be better utilized than its precursors. The actual contribution from dairy products depends, of course, on local consumption and vitamin potency of the fat. McGillivray and Thompson (1959) quote figures ranging from 20 per cent in Great Britain to just over 50 per cent in New Zealand.

The contribution from eggs is considerably smaller; in New Zealand, for example, about 10 per cent of our total vitamin-A requirement. Nevertheless, this is a significant quantity from a single food, and there is, again, the advantage of ease of absorption and high proportion of preformed vitamin. It is of interest to note that milk and eggs, produced under the best farming conditions, each contain about the same vitamin-A potency in their fat—50–60 I.U. per gramme.

2. The Young Animal

Placental transfer of vitamin A from mother to young is very limited, and foetal liver and blood vitamin levels are normally extremely low. Only when pregnant animals receive massive doses of vitamin A does the passage to the young via the placenta become significant (*e.g.* Walker *et al.* (1949) for data on calves). Thus, under the usual conditions obtaining in practice, although the maternal diet may be quite adequate for the animal's own requirements, the young may be born with blood vitamin-A levels only 10–20 per cent of those normally found in the adult, and with little or no vitamin reserve detectable in the liver. In these cases the offspring are almost entirely dependent on an intake of vitamin A from colostrum and early milk to meet their daily requirements and to build up reasonable liver reserves. The effect of suckling colostrum is immediately apparent on the vitamin-A status of the young animal. Four- to five-fold increases have been reported in the vitamin-A levels of the blood of calves 24 hours after suckling, and the increases in blood carotenoids are sometimes even more spectacular (*e.g.* Moore and Berry, 1944). The significance of carotenoid absorption at this early stage is, however, questionable, since there are indications (*e.g.* Domanski, Dobrowolska and Zalewska, 1956) that the ability of young animals to convert carotene to vitamin A is very limited. In any event, high intakes of vitamin A tend to depress carotenoid utilization, so that the value of colostrum, so far as its vitamin-A potency is concerned, most probably lies mainly in its preformed vitamin content.

This raises the whole question of the value of carotenoids as sources of vitamin A when fed along with preformed vitamin, as, for example, in milk. As already mentioned, the uptake of carotene from milk, by calves, seems very limited, their blood containing little carotene until after they have access to pasture. Where β-carotene and vitamin A occur together in a food it is usual to express the total vitamin-A potency as the sum of the carotene and vitamin A content, both expressed as International Units, using the generally accepted factors of 1 μg β-carotene = 1·67 I.U. and 1 μg vitamin A = 3·33 I.U. It is assumed that these factors, which really relate to β-carotene and vitamin A fed independently and under specified conditions, can be applied to a mixture of the two to give a measure of the available vitamin A. To the author's knowledge this point has not been verified, and more precise information on the utilization of carotene in the presence of preformed vitamin A would seem fundamental to much of our work on human and animal nutrition.

With birds, a different situation exists. The newly-hatched chick has not available to it any vitamin-A rich source such as colostrum, and is, therefore, dependent on the vitamin content of the egg yolk,

not only during the incubation period but for some time after hatching.

B. Factors Influencing the Vitamin-A Potency of Milk

The most extensive investigations into factors affecting the levels of carotenoids and vitamin A in milk have related to the bovine. In general, milk carotenoid levels tend to reflect blood levels, so that factors already discussed as influencing the carotenoid content of the blood will, in turn, affect milk levels. Tarassuk and Regan (1943) established a direct relationship between carotene levels in blood and the corresponding milk fats, but their formula is probably of limited application since, under New Zealand conditions at least, blood levels are considerably higher than would be expected from this relationship. The mammary gland shows a differential permeability to carotenes and xanthophylls, milk fat being relatively much richer than blood in the latter.

The vitamin A in the milk of most species is mainly in the esterified form (Thompson *et al.*, 1949*b*). The amount present bears no direct relation to total blood vitamin A, but appears to be fairly closely correlated with blood vitamin-A ester, from which it is probably mainly derived (*see* p. 150). Factors which influence carotenoid absorption will therefore tend to affect not only the level of carotenoids but also of vitamin A in the milk fat.

1. Effect of Breed

The carotenoids show greater variations between breeds than vitamin A, but the total vitamin-A potency remains relatively constant. Typical findings, relating to cows of different breeds kept, within each group, under the same conditions of feeding and management, are shown in Table 6.1. Considered in relation to the work, discussed later, on the origin of milk carotenoids and vitamin A, this tendency for between-breed variations in carotene content of milk fat to be offset by changes in vitamin-A level to give a relatively uniform total vitamin-A potency, would seem to indicate that it is a more efficient conversion of carotene to vitamin A rather than decreased carotene absorption, which is responsible for the relatively lower blood carotenoid levels in certain breeds. In these cases, the blood chylomicrons contain more vitamin-A ester in transport from the intestine, and correspondingly less carotene.

TABLE 6.1

Carotenoid and Vitamin-A Content of Milk of different Breeds of Dairy Cattle (per gramme fat)

Country	Breed	Carotenoids µg	Vitamin A µg	Total vitamin-A potency (I.U.)
United States winter milk-fat	Guernsey*	5·8	6·4	31
	Holstein*	3·4	7·3	30
	Ayrshire*	4·1	7·8	33
G. Britain winter milk-fat	Guernsey†	13	3·4	33
	Shorthorn†	5·4	5·5	27
New Zealand summer milk-fat	Jersey‡	9·3	7·5	41
	Friesian‡	5·8	8·9	39

* Wise *et al.* (1947). † Thompson *et al.* (1949*b*).
‡ Barnicoat (1947).

2. Stage of Lactation

Colostrum may be 10–20 times richer in carotene and vitamin A than normal milk (Walker *et al.*, 1949; Barnicoat, 1947; Thompson and McGillivray, 1957; McGillivray *et al.*, 1959). However, levels decline rapidly, and by the fourth day, when the milk is first used for human consumption, both carotene and vitamin A are becoming normal, and it is generally accepted that there are no further lactational trends (Hibbs, Krause and Monroe, 1949; Barnicoat, 1947). The actual levels of carotenoids and vitamin A, in the first colostral samples drawn, are related to the blood levels obtaining some two to three weeks previously, and are virtually independent of immediate dietary intakes and blood contents at parturition (Spielman *et al.*, 1947; McGillivray *et al.*, 1959). The rate of decline from colostral to normal milk levels is, however, dependent on immediate dietary intakes.

In assessing the value of colostrum to the calf it must be remembered that fat production is relatively low over the period when the colostral fat is richest in carotene and vitamin A. Nevertheless, the average secretion of these substances in the colostrum over the first few days of lactation is at least 50 per cent above normal (Thompson and McGillivray, 1957).

3. Effect of Feed

Variations in the carotene intake of cows are responsible for the greatest variations in the vitamin-A potency of milk (Booth *et al.*, 1933; Lord, 1945; Cary, 1947; Thompson *et al.*, 1949*b*). A number of workers have attempted to relate carotene intake to milk fat potency. Although lower figures have been set, most workers would now agree that to produce milk fat

of maximum potency the pasture must contain at least 26 mg β-carotene/100 g dry matter (Hibbs *et al.*, 1949) giving a daily intake in the order of 2·5 g carotene (Wiseman, Shepherd and Cary, 1949). Below this point there is a fairly direct relationship between carotene intake and milk fat vitamin-A potency, but above it, additional carotene causes no increase in the quantities of carotene or vitamin A present in the fat.

This dependence of vitamin-A potency of milk fat on immediate carotene intake is responsible for marked seasonal changes in the potency of dairy products produced in most parts of the world, values tending to be low during winter stall-feeding periods, and higher when the cows are out-of-doors on pasture. In summing up the voluminous literature on this subject, we may say that the vitamin-A potency of market milk or milk products in various parts of the world is a reflection of feeding and management in these areas, modified by the characteristics of the dominant breed. As an example of the type of variation encountered, Table 6.2 (after McDowell and McDowall, 1953) shows the vitamin-A potency of winter and summer butters from various parts of the world.

TABLE 6.2

Comparison of Vitamin-A Potencies of Butter in different Countries

Country	Vitamin-A potency expressed as			
	I.U./g		I.U./lb	
	Winter butter	Summer butter	Winter butter	Summer butter
New Zealand	41·9	33·8	19,010	15,330
Great Britain	18·5	29 0	8,390	13,150
Sweden	15 0	24·2	6,800	10,980
Denmark	16·5	33·1	7,480	15,010
United States*	21·0	34·3	9,500	15,600

* Re-calculated from values published by Cary (1947), using the factor of 3·33 now accepted as correct for the conversion of vitamin A expressed in μg into international units and assuming that 20 per cent of the potency is due to carotene.

C. Variations in the Vitamin-A Potency of Milk from Cows on Pasture

The carotene intake of pasture-fed cows is usually considerably in excess of the figures quoted above, for the production of milk fat of maximum vitamin-A potency. Under conditions of year-round pasture feeding, such as those obtaining in New Zealand, a uniformly high vitamin-A potency might be expected. That this was not the case was first reported by Barnicoat (1947), who found marked seasonal variations in butters representative of two dairying districts during the 1935–36 season. Both carotene and vitamin A were affected, and the variations were in the opposite directions to those reported for overseas butters, the maximum potencies occurring in late winter and early spring, and the minimum values in mid-summer. These changes were shown to be seasonal rather than lactational, and were confirmed by McDowell and McDowall (1953) in a more extensive investigation covering other New Zealand dairying districts, and extending over several seasons. Fig. 6.1 compares the trend in New Zealand (after McDowell and McDowall) with that reported in Great Britain by Lord (1945). Similar seasonal fluctuations in carotene and vitamin-A levels have been reported by Farrer *et al.* (1949) in districts in Australia where dairying conditions are similar to those in New Zealand. There is, also, some indication of a similar mid-summer decrease in results reported by Thompson *et al.* (1949*b*) for butter from the south-west of England.

A decreased carotene intake associated with the drying up of the pasture over the summer months would seem the most logical explanation for these seasonal variations, but typical dairy pastures in New Zealand provide lush feed of high carotene content throughout the year, giving a daily intake for dairy cows considerably in excess of the 2·5 g quoted above. The problem would appear, therefore, to be one of varying carotene utilization rather than intake, some factors reducing the availability of carotene from summer pastures. It is significant that similar and closely related seasonal trends are apparent in the iodine value of the milk fat and in its tocopherol content (McDowell and McDowall, 1953; McGillivray 1956). It would appear, therefore, that the factors reducing carotene absorption also influence the absorption of other fat-soluble materials such as tocopherol, and interfere with fat absorption or metabolism in general.

In so far as the fat-soluble vitamins are concerned, the mid-summer decreases in the milk fat are merely reflections of changes which occur in the levels of these substances in the blood plasma of cows on pasture (McGillivray, 1957*b*). Extensive investigations have so far failed to provide a complete explanation for these variations in fat-soluble vitamin absorption. The effect of factors other than carotenoid intake was clearly demonstrated in a series of experiments in which the proportion of fresh pasture in the diet of lactating cows was progressively reduced (McGillivray

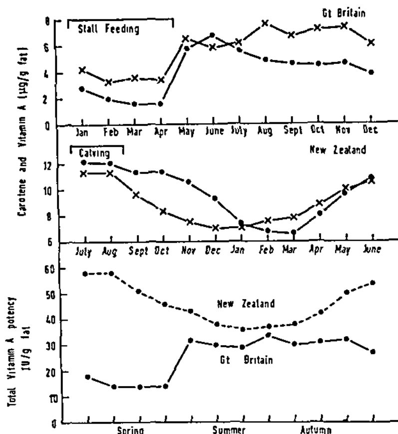


FIG. 6.1. COMPARISON OF CAROTENE AND VITAMIN-A CONTENT AND TOTAL VITAMIN-A POTENCY OF MILK FAT PRODUCED IN GREAT BRITAIN (after *Lord*, 1945) AND IN NEW ZEALAND (after *McDowell and McDowell*, 1953)

●——●, carotene; X——X, vitamin A; - - - -, total vitamin-A potency; full line, Great Britain; dotted line, New Zealand.

The results for Great Britain relate to a herd of Ayrshires, whereas those for New Zealand represent the fats from commercial butters from mixed herds, predominantly Jersey. The effect of reduced carotene intake during the period of stall feeding in Great Britain is apparent, the variation in carotene and vitamin-A content of New Zealand milk fat is, however, unrelated to changes in the actual quantity of carotene in the pasture

and Worker, 1957*b*). The carotene intake from full pasture was approximately 5 g/day, and the corresponding vitamin-A potency of the milk fat 36 I.U. Reduction of the carotene intake to 2·5 g/day had no effect on the vitamin A in the milk, and depressed the carotene by only about 1 µg/g fat. A slight effect on milk fat vitamin A was apparent when the daily carotene intake was reduced to 1·3 g, and this was associated with a further decrease of about 1 µg/g in milk fat carotene. Dropping the carotene intake below this level caused marked reductions in both vitamin A and carotene in the milk fat.

Thus, although the generally accepted daily carotene intake for production of milk fat of maximum vitamin-A potency is about 2·5 g, these cows seemed incapable of utilizing more than about 1·3 g from the summer pastures, which consisted of the ryegrass (*Lolium perenne*)–white clover (*Trifolium repens*) association typical of many dairying areas of New Zealand.

A striking feature of this, and other trials with pasture-fed cows, has been the magnitude of the day-to-day variations, particularly in the carotene content of the milk fat, during periods when the carotene intake from pasture remained constant. These variations often exceed experimental differences and, despite wide differences in treatments, are always closely correlated in control and experimental cows. It would seem that environmental factors may be

of maximum potency the pasture must contain at least 26 mg β-carotene/100 g dry matter (Hibbs *et al.*, 1949) giving a daily intake in the order of 2·5 g carotene (Wiseman, Shepherd and Cary, 1949). Below this point there is a fairly direct relationship between carotene intake and milk fat vitamin-A potency, but above it, additional carotene causes no increase in the quantities of carotene or vitamin A present in the fat.

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carotene, but these substances are found, as in normal milk, mainly in the fat globules.

McGillivray has further suggested, however, that, in all milks, a small proportion of the vitamin-A alcohol and carotene remains associated with those milk proteins which may be merely transudates of blood, or becomes associated with other milk proteins during the course of synthesis in the mammary gland. Where the uptake of blood proteins is higher than usual, for example at the onset of lactation or with diseases of the udder such as mastitis, the carotene and vitamin A content of the milk is also increased and a higher proportion remains associated with the proteins. Thus, the proportion of protein-bound vitamin and pro-vitamin is highest in pre-partum secretions, and decreases through the colostral period to normal milk where only relatively small amounts are present.

On this basis, therefore, it would seem that the vitamin-A potency of milk depends, primarily, on the carotene and vitamin-A content of the chylomicrons and, to a lesser extent, on the uptake of certain plasma proteins by the mammary gland. The high levels, then, of fat-soluble vitamins in colostrum are merely a secondary effect of the increased uptake of plasma proteins (McGillivray, 1959). This theory for the origin, not only of carotene and vitamin A, but also of other fat-soluble substances in milk, explains relationships observed under normal and abnormal conditions between intake, blood levels and milk content of these materials.

E. The Carotenoid and Vitamin-A Content of Eggs

Hens carry into their egg yolks at least a part of any carotenoid fed to them. However, irrespective of the ratio of carotenoids fed, xanthophylls make up the largest portion of the carotenoids in the egg, carotene being present only to the extent of about 10 per cent or less (*e.g.* Mann, 1946). Lutein is the principal xanthophyll, making up about 70 per cent of the total carotenoids in the eggs of hens on common rations. The remaining pigment is mainly zeaxanthin (Kuhn, Winterstein and Lederer, 1933). Thus, whereas the ratio of carotenes to xanthophylls is in the order of 1:2 to 1:3 in normal green feeds, the proportion in hens' eggs is about 1:10 and may even reach 1:30 (Goodwin, 1952). This is thought to be due to the efficient conversion of absorbed precursor carotenes to vitamin A, rather than to any preferential absorption of xanthophylls.

Vitamin A is normally present in egg yolks, the amount varying with the diet and, for well nourished hens, averaging about 15 μg/g or 240 μg/yolk (Vermes, Meunier and Raoul, 1939). Most of the vitamin A is in the free alcohol form (Neff *et al.*, 1949).

In general, diet influences the carotenoid and vitamin-A content of eggs much as it does that of milk. There is a relationship between dietary carotenoids and the colour of egg yolk, and when hens are placed on a carotenoid-free diet, the colour of their yolks gradually decreases.

Although the vitamin-A requirements of poultry are most conveniently supplied by the addition of small amounts of cod liver oil in the diet (usually 1 per cent) or, as is more common now, equivalent amounts of synthetic vitamin A in the form of stable "dry mixes," consumer preferences for reasonably highly coloured egg yolks necessitate the inclusion of some carotenoid source such as dried lucerne meal. It is generally recognized, however, that even liberal access to fresh grass is not an adequate substitute for preformed vitamin A in the diet. As with most species, this preformed vitamin A interferes to some extent with carotenoid absorption, but at the levels of vitamin A normally fed, the reduction in carotenoid content, hence colour, of the egg yolks is not of practical significance (Deuel *et al.*, 1943). The preference for coloured yolks is purely an aesthetic one since, due to the high proportion of non-precursor pigments, yolk colour is no index of vitamin potency.

Neither the carotenes nor the xanthophylls in egg yolks are metabolized during incubation (Suomalainen 1939; Mann, 1946) but, for the hatching to be successful, the egg must contain at least 100 μg vitamin A (Lissot and Caridroit, 1941), and a fair proportion of this appears in the body of the newly-hatched chick. The effect of differences in the vitamin-A content of the hens' diet on the amount of vitamin A transferred to the embryo chick becomes increasingly apparent as incubation proceeds (Parrish *et al.*, 1950) and indicates, once again, the importance of considering not only the immediate requirements of the adult, but also the needs of the young offspring, in the rationing of livestock.

F. Carotenoids and the Colour of Animal Products

Every housewife knows the important part played by colour in determining the acceptability of foods. In animal products such as butter, depot fats and eggs, very marked and long-established colour preferences are apparent. These differ very considerably in different parts of the world and would seem to be determined largely by the nature of the products traditionally available in each area. On the one hand, there is the older, and, of course, largely erroneous, idea that the rich yellow colour of Jersey milk or the deep orange-red of farm eggs is necessarily associated with, and indeed the only index of, high nutritive value in these foods. In other areas there is the demand for almost white butter, and the rejection of meats in which the

involved, and these might also operate to produce a general lowering of carotene utilization over the summer period. No simple correlation with climatic changes, however, has been established.

Working on the assumption that tocopherol might be lacking in summer pasture, McGillivray (1952) demonstrated the beneficial effect of supplements of this vitamin, but his work, and subsequent studies on pasture composition (Worker, unpublished results) does not conclusively establish tocopherol as a factor associated with the seasonal variation in the vitamin potency of milk fat.

Over the summer period, clover emerges as the dominant species in many New Zealand pastures. Investigating the effect of botanical composition of the pasture, Worker and McGillivray (1957) showed that the ingestion of high-cyanide, white clover resulted in decreased milk fat vitamin-A potencies. Non-cyanogetic red clover (*Trifolium pratense*) did not have the same effect, but the cyanogetic glycoside in the white clover did not appear to be the factor involved. Further work (McDowall and McGillivray, unpublished results) has confirmed the apparent influence of botanical composition, but the results are not as clear-cut as those of Worker and McGillivray. It would appear that the stage of maturity of the pasture species is an important consideration, and McDowall and McGillivray have found that slight maturing of the pasture, although it is still in a lush condition with a high carotene content, results in marked decreases in the carotenoid content of the blood and in the vitamin-A potency of the milk fat of the cows.

Lush, high quality pasture, as it is grown throughout the year in countries such as New Zealand, provides a luxury supply of carotene to grazing animals. Clearly, however, we know little about the way in which they are able to utilize it. It would seem that a number of factors, including those already discussed, are involved, not only in carotenoid absorption, but also in the utilization of other fat-soluble vitamins and, probably, of pasture fat itself. These factors may effect absorption directly or, since all the studies have been on ruminants, they may exert a more indirect influence through changes in rumen activity associated with the different microfloral populations stimulated by the changing composition of the feed. The problem is a complex one.

D. The Origin of Milk Carotenoids and Vitamin A

During the course of the work on the utilization of carotene from pasture by lactating cows, just discussed, the need for more precise information on the immediate origin of milk fat carotenoids and vitamin A became apparent. It is well recognized that, for the production of milk fat of high vitamin-A potency, cows are more dependent on a daily carotene or vitamin-A intake than on liver reserves of vitamin A (*e.g.* Hjarde, Nielsen and Porotnikoff, 1954). Both the carotene and the vitamin-A contents of the milk fat respond very rapidly to changes in vitamin or provitamin intakes, irrespective of body reserves, but at the same time, for example during periods of severe vitamin deprivation, there is evidence of some contribution from liver and body stores of carotene and vitamin A to milk fat levels.

In the case of vitamin A we have, in the blood, the two chemically and physically distinct forms: esterified vitamin in transport from the intestines to the storage organs; and free vitamin-A alcohol, in the form of a protein complex, maintained at a relatively constant level from liver reserves. The quantity of vitamin A in the milk must be related to the amounts of these two forms in the blood. McGillivray (1957*c*) considers that we must postulate the existence of two analogous, physically distinct, forms of carotene in the blood; one, a normal circulating form making up the bulk of the carotene in the blood plasma, corresponding to the plasma vitamin-A alcohol and, like it, probably intimately associated with plasma proteins; and the other, a transport form, corresponding to plasma vitamin-A ester, and present when the animals are on a carotene-containing diet, and at levels which reflect the immediate carotene intake.

This transport form of carotene, together with vitamin-A ester and other fat-soluble materials of immediate dietary origin, is probably associated with dietary fat in the chylomicrons whose uptake by the mammary gland (*e.g.* review by Glascock, 1954) provides the bulk of the fat-soluble vitamins appearing in milk fat. The association of these substances with dietary fat in the chylomicrons taken up by the mammary gland would explain the close correlations established between carotenoid, vitamin A and tocopherol content of milk, and the iodine value of the fat.

The contribution of body stores to milk vitamin levels must derive through an uptake, by the mammary gland, of protein-bound vitamin-A alcohol and carotene from the blood. There is evidence (McGillivray, 1957*d*) that these materials are taken up by the gland along with plasma proteins. During the changes which occur in the cells of the gland, much of this vitamin-A alcohol and carotene must become dissociated from the proteins and be transferred to the fat, the vitamin-A alcohol being fairly completely esterified in the process. This change from protein association to fat phase is apparent in cows on a diet devoid of carotene and vitamin A. Here, the protein-bound vitamin-A alcohol and carotene of the blood provide the only source of milk vitamin A and

since the ratio of feed intake to body weight increases with decreasing size. This system would seem to have considerable merit. It is an extension of the method normally used for poultry where vitamin-A requirements are usually expressed as a minimum content in the feed (*i.e.* I.U. vitamin A per pound).

It must be remembered that the above recommendations relate only to the animals' own requirements, and that most practical tables make an extra allowance for conditions such as pregnancy and lactation. These are, however, relatively small, and it is, perhaps, not too much to hope that in the assessing of vitamin requirements of animals, greater attention might be paid to their effect on the nutritive value of animal products such as milk and eggs used for human consumption.

B. Deficiency Symptoms

Any detailed discussion of the diversity of pathological conditions that have been attributed to vitamin-A deficiency in domestic animals is beyond the scope of this review. Suffice it to say that damage is widespread but, in the most general terms, may be considered as changes in epithelial tissues, bones and nerves. Indeed, if we are to see progress in this field, we must switch our thinking from the multiplicity of deficiency symptoms to a simplification of the picture in terms of specific effects of vitamin A within certain cells.

Stratification and keratinization of epithelial tissue is apparent in the respiratory, alimentary, reproductive and genitourinary tracts as well as in the eye. This may, in turn, lead to secondary infections which do not necessarily respond to vitamin-A therapy. It may also result in other conditions of a non-infective character such as gastro-intestinal disturbances, the initiation of urolithiasis through sloughed keratinized cells forming nuclei for stone formation, etc. It is responsible for specific reproductive failures; in the male there is a degeneration of germinal epithelium of the testes resulting in a decreasing and, in severe deficiency, eventual cessation, of spermatogenesis; in the female, cornification of the vaginal epithelium produces irregular estrus and, together with degeneration of the placenta, premature termination of pregnancy or congenital abnormalities in the offspring.

Thus, although still lacking the knowledge of the precise biochemical action involved, we may ascribe a wide variety of apparently unrelated deficiency symptoms to the one function of vitamin A in maintaining the normal condition of epithelial tissue.

The effect of vitamin-A deficiency on bones and nerves is less clearly defined. In the absence of vitamin A, increased and disorganized activity of the osteoblasts and osteoclasts results in abnormalities in bone formation and modelling. A secondary effect of these bone malformations may be nerve degeneration following constrictions of bone canals and resultant pressure on nerves. Thus, bone changes may be responsible for the muscular inco-ordination and other nervous symptoms typical of vitamin-A deficient animals. At the same time, there is also evidence of a more direct effect of vitamin-A deficiency on nerve structure.

Perhaps nowhere more than in the eye is the complexity of vitamin-deficiency better demonstrated. The earliest symptom, night blindness, is a simple one, vitamin A being non-available for rhodopsin synthesis, and the condition readily responds to therapy. Epithelial breakdown, affecting the cornea and conjunctiva, may be followed by infections which can lead to permanent blindness through damage to the cornea or lens. Alternatively, more sudden blindness may result from degeneration of the optic nerve, possibly as a result of abnormalities in bone formation (Moore, 1957).

These and other symptoms have been reported in domestic animals under experimental conditions and sometimes also under practical farming conditions. As pointed out in the introduction, however, better animal husbandry has led to the virtual disappearance of these gross symptoms. When we do encounter vitamin-A deficiency under practical conditions, the symptoms, in this country at least, are usually mild and almost sub-clinical. They lack specificity, and if they are, in fact, in any way associated with vitamin-A deficiency, are frequently complicated by other dietary shortcomings. In these cases it is fortunate that our knowledge of vitamin-A requirements is such that diagnosis, from the nature of the diet, of possible vitamin-A deficiency is usually a relatively simple matter. Despite the wide spectrum of primary and secondary deficiency symptoms, vitamin A is no "cure-all," and there is no justification for the all-too-common practice of "shot-gun" therapy, including carotene, vitamin A, and most other minor nutrients, in those cases where the nature of the diet precludes any possibility of deficiency: its only merit lies in the fact that it probably does no harm and may, perhaps, in some chance way, add something to our all too incomplete knowledge of the role of carotene and vitamin A in animal feeding.

fat is at all coloured. As markets have become more competitive these preferences have tended, over recent years, to focus attention on factors which control the deposition of carotenoids in these products.

An adequate supply of xanthophylls in the diet of laying hens, for example, will ensure a reasonable colour in egg yolks, but deposition of carotenoids is not limited to the egg, and the associated yellowing of the body fat may detract from the bird's subsequent value as dressed poultry. Carotenoid absorption by bovines constitutes an even greater aesthetic problem, particularly where animals are pasture fed throughout the year. Although the actual concentration of carotenoids in the body fat of most breeds is not particularly high, it increases quite markedly with age and presents a marketing problem with older beef cattle and, particularly, with cull dairy cows. In New Zealand, the predominant Jersey breed, the high carotene intake, and the concentration of calving over a short period in the early spring, all contribute to the production, at least over these spring months, of milk fat of high carotenoid content. The resultant fairly intense yellow colour of the butter is frequently stated to be a major factor prejudicing its sale overseas. Indeed, one economic advantage claimed for the use of heavy liquid paraffin in bloat control (*see* pp. 138-9) is the associated reduction in the yellow colour of butter.

These are very real economic problems related to carotenoid metabolism but, since they involve the moulding of public opinion rather than altering the product itself, their solution would seem to lie in the hands of the advertiser and educationalist rather than with the nutritionist. It seems paradoxical that, while there is interest in New Zealand in reducing the natural carotene content of butter, increasing use is being made elsewhere of synthetic carotene as a butter-colouring agent.

VI. THE VITAMIN A REQUIREMENTS OF DOMESTIC ANIMALS AND GENERAL SYMPTOMS OF VITAMIN-A DEFICIENCY

A. Requirements

Although carried out over 20 years ago, important studies on the vitamin-A requirements of domestic animals by Guilbert, Miller and Hughes (1937) still form the basis of our vitamin-A rationing of cattle, pigs and sheep. The technique used by these workers was to maintain the animals on diets low in vitamin A until they developed night blindness, as indicated by their inability to avoid obstacles in semi-darkness. The daily allowance of vitamin A or carotene necessary to restore normal dark adaption was then determined by giving varying doses over periods of a few weeks up to several months. Allowance was made for any vitamin A reserves which had accumulated during the trial, and minimum daily allowances of carotene or vitamin A were calculated.

These results, and subsequent work on the requirements of the horse (Guilbert, Howell and Hart, 1940) have been reviewed by Hart (1940), who made allowance for the corrected potency of the cod liver oil used as a source of vitamin A. His oft-repeated figures still bear repetition here:

Species	Daily requirement per kg body weight (I.U.)	
	Vitamin A	Carotene
Cattle	21–27	43–55
Sheep	17–26	42–58
Pig	18–24	42–65
Horse	17–22	30–50

Hart regarded these as minimum figures and recommended, in practice, intakes of 5 to 10 times these amounts. In this light, the small species differences in the table above are relatively insignificant, and a reasonable practical interpretation of this work would seem to be a recommendation of daily intakes of from about 100–200 I.U. of vitamin A or 240–480 I.U. carotene per kg body weight. This simplification is in line with modern rationing practice and current recommendations, for most species of domestic animal, of about 6 mg carotene per 100 lb body weight (*e.g.* Morrison, 1957; Woodman, 1957) represent intakes of just under five times Hart's average minimum figure for carotene.

Hart supported the conclusion that the vitamin-A requirements of domestic animals of varying sizes were proportional to their body weights, and not to energy expenditures. Moore (1957), however, takes a somewhat contrary view and, comparing recommended allowances for humans and for rats, shows that the body weight relationship does not apply and that a more satisfactory comparison is on the basis of relative feed intakes. Thus, on body weight, 2,500 I.U. for a human is equivalent to only 7 I.U. for the rat, a figure known to be too low; but comparison in terms of calorie intake gives the much more acceptable figure of 63 I.U. for the rat.

When dealing with animals of comparable size, the distinction between the two systems can probably be neglected. Within any one species Moore's system would have the effect of increasing, relatively, the vitamin allowance for younger and smaller animals,

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7

Parathyroid Glands and Calcium Metabolism

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Contents

I. INTRODUCTION

We are now entering a phase of parathyroid research which compares almost in importance to that opened up by Collip (1925) and by Greenwald and Gross (1925). The year 1925 saw the preparation of the first active extracts of the parathyroid glands, and the demonstration of the calcium-mobilizing and phosphaturic activities of the hormone. In the intervening years the physiology of the parathyroids has been elucidated so far as has been possible with the techniques and tools available. Many an experiment has been based on observing effects elicited by administration of commercial parathyroid extracts, which are at the most only 2–3 times purer than crude extracts. In such studies, interpretation is uncertain, due to the difficulty of distinguishing between effects which are hormonal and those which are non-specific. As a consequence the pendulum has swung, in many instances, between two extremes: for example, the concept that the phosphaturic activity of the hormone is its primary physiological action (Albright and Reifenstein, 1948) has contrasted the opinion that the phosphaturic activity is entirely artifactitious (Stewart and Bowen, 1952). The solution of such problems will be made easier by the advent of the pure hormone or hormones.

The current phase of parathyroid research is marked by the preparation, in more than one laboratory, of parathyroid material which, perhaps not yet a pure hormone, is 250 times purer than crude extracts. When these preparations become available in larger quantities a new phase of parathyroid biochemistry and physiology will undoubtedly begin. The availability of purified material is coming at a time when the biochemistry of parathyroid hormone action is beginning to receive the attention it deserves. To attempt an *in vitro* biochemical study with the crude extracts available heretofore is fraught with too many difficulties of both technique and interpretation to merit even consideration.

Recent events in the area of calcium nutrition deserve special mention because of the newer data and changing attitudes towards the recommended level of calcium requirement for man. Hegsted, a doughty contestant in the tourney of calcium nutrition, has recently been strengthening his experimental armour and sharpening his verbal lance.

The relationship between the parathyroids and phosphorus metabolism, a topic difficult to dissect out from that under review, has largely and reluctantly been excluded from the discussion as being inappropriate. For a similar reason the interrelationships between vitamin D, the parathyroids and calcium metabolism have been alluded to only where necessary. The whole important area of the dynamics of calcium metabolism, as approached with the aid of radiocalcium, has been neglected in this review, for this has been well covered recently by Neuman and Neuman (1958). The rapidity and relative completeness with which injected calcium enters the bone, the fact that endogenous calcium is released into the gastro-intestinal tract and is lost in the faeces, these and certain other problems have succumbed to solution by the use of the radiocalcium technique. However, the present author agrees with Neuman and Neuman (1958) that the kinetic approach to calcium dynamics, in which the observed curve for the rate of disappearance of injected radiocalcium from the blood is broken down into a series of exponential curves, each assigned a physiological meaning or compartment, has been disappointing. The method is rife with complexities, a fact which led Bauer, Carlsson and Lindquist (1955) to propose a simpler approach to the problem.

II. CHEMISTRY AND PHYSIOLOGY OF THE PARATHYROIDS

A. Chemistry of Parathyroid Hormone

One of the most significant advances in parathyroid research within recent years has been the rapid progress made in the purification of the hormone. By 1955 the stage had been set for such a development by the publication of more convenient and reasonably precise methods for the assay of the hormonal activities of parathyroid extracts (Munson, 1955; Kenny, Vine and Munson, 1954; Davies, Gordon and Mussett, 1955). Preparations of the purified hormone are now available which represent in terms of units/mg protein at least a 200-fold purification of crude extracts. The best potencies reported from the laboratories which have been most active in this field are 1000 units/mg protein (Munson and Voelkel, quoted in Kenny, 1959), 900 to 1,200 units/mg (Rasmussen, 1959*a*), and 500 units/mg (Aurbach, 1959*c*). Rasmussen started with the ultrafiltration procedure of Davies and Gordon (1955), and subsequently made use of such techniques as displacement chromatography on Dowex 50 resin (Rasmussen and Westall, 1957*a*), electrophoresis on a solid medium,

polyvinyl chloride (Rasmussen, 1957), and counter-current distribution (Rasmussen, 1959*a*). The conditions of the initial extraction procedure were found by Rasmussen and Westall (1957*a*) to affect notably the subsequent behaviour of the activity. Although extraction of the fat-free glands with 0·2 N hydrochloric acid gave a crude extract which responded well to subsequent ultrafiltration and displacement chromatography procedures, an extract prepared with acetic acid responded less well to ultrafiltration and failed to respond to displacement chromatography. These differences between extracts have led Rasmussen and Westall (1957*a*) to distinguish them by name; they called the hydrochloric acid material, Parathormone A (PTH-A), and the acetic acid material, Parathormone B (PTH-B). It was only the later material which was subjected to further purification by means of electrophoresis. It was, in the course of these studies, that Rasmussen made, in the opinion of the reviewer, two very significant discoveries: the shorter duration of action and the instability of purified preparations. He became acutely aware of the instability of the purified hormone and made a separate study of this phenomenon (Rasmussen, 1958, 1959*c*). He found that the hormone could be largely inactivated by oxidizing agents such as hydrogen peroxide (confirming the earlier findings of Tweedy, Bell and Vincens-Rios, 1935) but, in addition, made the very important observation that the activity could be restored by treatment with a reducing agent, cysteine, at 80°C for 6 hours. The latter finding has been confirmed by Kenny (1959), who noted that addition of cysteine to a crude parathyroid extract at a concentration of 0 03 M at room temperature was somewhat better under his conditions than the procedure recommended by Rasmussen (1958, 1959*c*). It is not clear from the data presented by Rasmussen (1958, 1959*c*) whether or not the initial activity of the hormone was assayed in the presence of cysteine, for, in a separate publication, Rasmussen (1959*b*) reported that cysteine will enhance the activity of Parathormone B which had not been intentionally inactivated in any way. Thus, the recovery of the activity resulting from treatment with cysteine of the hormone which had been inactivated with hydrogen peroxide does not necessarily mean that the cysteine is specifically reversing the inactivating effect of hydrogen peroxide. It may be enhancing the activity of that portion of the hormone which was not destroyed by the hydrogen peroxide. Such an interpretation would be consistent with the data, which indicated that the less the hormone is inactivated the greater the recovery of the activity. More conclusive evidence, such as the isolation of the hydrogen peroxide-inactivated material from the active material, and the demonstration that the former material could be reactivated by cysteine, is required to demonstrate the presence and biological importance of an oxidation-reduction site in the hormone molecule. It is perhaps significant that other reducing agents such as stannous chloride, hydrogen sulphide, ascorbic acid and sodium borohydride were ineffective in restoring the activity of oxidized Parathormone B (Rasmussen, 1959*c*). In connexion with the effect of cysteine, Kenny (unpublished observations) has shown that a chelating agent, 2,2'-bipyridine, completely antagonized the activating effect of cysteine. This phenomenon suggests that a metal ion is necessary, perhaps as a catalyst or as part of the hormone molecule, for the activation of parathyroid hormone by cysteine.

The demonstration, also by Rasmussen (1959*b*), that purified preparations gave a markedly different time-response relationship in the calcium-mobilizing assay, is a further noteworthy contribution. Whereas the blood calcium of parathyroid-ectomized rats, when injected with crude parathyroid extract, reached a maximum in 14–18 hours, with a purified preparation it attained the peak at 5–7 hours, and had returned to control levels within 12–14 hours. This observation has been one of the factors contributing to the preference, by those currently engaged in purification studies, for the calcium-mobilizing assay method of Munson (1955), which employs a 6-hour interval between the injection and the determination of the serum calcium. Further safeguards against this problem may be taken by the use of various injection media which increase and/or prolong the action of the hormone; arachis oil and heparin (Rasmussen, 1959*b*) and gelatin (Rasmussen, 1959*b*; Aurbach, 1959*a*) have proved effective in this respect. Cysteine, of course, may be included in this category, although its action is probably more complex than a simple retardation of absorption from the injection site.

Friedman and Munson (1958*a*, *b*) and Munson (1959) have employed a simple two-stage procedure consisting of precipitation of the crude extract with ammonium sulphate (0·3 to 0·4 saturation fraction) and subsequent elution with increasing ionic strength of sodium chloride from a carboxymethyl cellulose column. In Astwood's laboratory, extraction with phenol and purification by counter-current distribution has been the method of choice (Aurbach, Beck and Astwood, 1958; Aurbach, 1959*b*).

It is now possible, with the hormone available in a greater state of purity, to obtain more reliable information concerning the probable chemical nature and biological activity of the hormone. However, there exist reports, in the recent literature from a group of French investigators, that the hormone is a steroid, which they claim has a hypercalcemic action

in intact rabbits (Raoul, Marnay and Prelot, 1955; Marnay and Prelot, 1957), in intact and parathyroidectomized dogs (Marnay, Prelot and Raoul, 1956; Marnay, 1957), and in parathyroidectomized rats (Marnay, 1957). Rasmussen and Westall (1957*b*) and Kenny (unpublished observations) have been unable to confirm the findings of Raoul and his associates. In addition, Rasmussen (1959*a*) could account for 96 per cent of the nitrogen content of his preparation by the amino acid analysis. Determinations of molecular weight have yielded values of 4,000–6,000 for PTH-A (Rasmussen and Westall, 1957*a*), and of 6,000–8,000 for PTH-B (Rasmussen, 1959*a*). This would mean that the parathyroid polypeptide is comparable in size to insulin, is larger than the other polypeptide hormones such as ACTH, vasopressin, oxytocin and glucagon, and is smaller than the protein hormones, growth hormone, prolactin and ICSH (Behrens and Bromer, 1958).

Since the widespread recognition that two hormonal activities are associated with the parathyroid glands, the question has been asked whether there are one or two hormones. This cannot yet be answered satisfactorily. Experiments with crude extracts, in which different ratios of calcium-mobilizing to phosphaturic activities were found, have been interpreted with caution as suggesting two hormones (Kenny and Munson, 1959). However, the purified preparations of Friedman and Munson (1958*b*) and of Rasmussen (1959*a*) exhibit each activity to a similar degree. This fact does not, of course, rule out the possibility of the existence of a separate, largely phosphaturic hormone; the preparations just mentioned were fractionated, using an assay for only the calcium-mobilizing activity, for the purpose of following the course of the activity.

It is tempting to speculate on the probable purity of the hormone preparations currently available. Rasmussen (1957) has subjected his PTH-B (210–230 units/mg) to some criteria of purity. It appeared homogeneous when exposed to zonal electrophoresis at pH 3·8 and to ultracentrifugation (S_{20}=1·47 Svedberg units); but investigators in the area of hormone purification, armed with the experience obtained with ACTH, are now extremely reluctant to claim absolute purity for their preparation even when it appears homogeneous by reputable criteria. As Munson and Voelkel (quoted in Kenny, 1959) and Rasmussen himself (1959*a*) now have preparations which are more than four times purer than Rasmussen's earlier preparation of PTH-B, the latter obviously cannot be the pure hormone. The picture is further complicated by the fact that PTH-A and PTH-B appear to be different chemically. Perhaps PTH-A represents a portion of the PTH-B molecule. Unlike Rasmussen's material, Munson's preparation was obtained without the use of heat at any stage (Munson, 1959); such a mild treatment would seem more desirable for the purpose of obtaining a homogeneous preparation.

The need for a parathyroid hormone standard, and standardized assay conditions, is becoming more acute if the activities of the preparations from different laboratories are to be compared. The three laboratories actively engaged in purification studies use commercial preparations as the standard in their bioassays, and although the three laboratories are basically using the assay method of Munson, it is not possible to expect that, if the same unknown were assayed in different laboratories, the determined potencies would be identical. Different batches of standard preparation and different injection media for the unknown would undoubtedly be used.

The important business of determining levels of parathyroid hormone activity in blood and urine has been neglected for a very good reason, the lack of a sensitive assay method. Davies (1958) has made a heroic beginning by measuring the phosphaturic activity present in human urine by means of her assay method in the mouse. This method proved sensitive enough for the assay of urines from normal (47–72 units/24 hr) and hyperparathyroid subjects (103–146 units/24 hr) but insufficiently sensitive for the detection of activity in the urine of patients with hypoparathyroidism (<30 units/24 hr). In any case it was necessary to use as much as a 48-hr urine sample for a single assay.

The application of one of the newer methods of purification for the initial extraction and isolation of the hormonal activity from urine or blood might prove more effective than the benzoic acid adsorption method used by Davies (1958). In this connexion an unpublished method of Kenny, which has proved effective for the initial purification of crude parathyroid extracts, might be useful. The method consists in applying the crude extract to a carboxylic acid type cation exchange resin (Amberlite, XE-97) at pH 4 and eluting the column with 0·1 N hydrochloric acid.

B. Physiology of Parathyroid Hormone

Before discussing in detail some of the current research trends in parathyroid physiology it would be appropriate to remind ourselves of a general problem in the study of endocrine physiology which is particularly applicable in the case of the parathyroids. The basic technique in endocrine studies is the extirpation of the gland, and the restoration of the *status quo* by injection of a glandular preparation. The success of this technique depends upon two main factors: (1) the completeness of the surgical removal of

the gland, and (2) the degree of purity of the hormone preparation. Mention has already been made of the latter problem, but careful consideration should also be given to the former, the thoroughness with which parathyroidectomy can be performed in experimental animals. Although probably not of much importance, in acute experiments the existence of accessory parathyroids would have considerable bearing on the interpretation of studies involving animals which were chronically parathyroidectomized. Greenwald and Gross (1929) had long believed in the impossibility of removing all parathyroid tissue in the dog, a suspicion which was confirmed by the anatomical studies of Godwin (1937). The problem of accessory parathyroid tissue is particularly noticeable in the chicken (Polin and Sturkie, 1958). Our complacency concerning this problem in the rat is based largely on the work of Hoskins and Chandler (1925), who were able to find only five instances of accessory parathyroid tissue in an anatomical study of 65 rats. That there is no difficulty in acute experiments is borne out by the experience in the laboratory of Dr. Paul Munson and that of the present author. In hundreds of control rats which have been parathyroidectomized following 4 days of calcium depletion, the serum calcium has consistently and significantly fallen within 6 hours, indicating a functional parathyroidectomy in each instance. However, in experiments with rats which had been parathyroidectomized for 1 week, Kenny (1960) has obtained unexpected results which may best be interpreted by assuming the existence of accessory parathyroid tissue in at least 80 per cent of the rats. These results are in agreement with the recent anatomical studies of Van Dyke (1959).

No time will be spent discussing the well-documented fact that parathyroid hormone has a direction action on bone, independent of its action on the kidney (Barnicot, 1948; Chang, 1951; Gaillard, 1955*a*, 1955*b*, 1957; Grollman, 1954; Greep and Kenny, 1955). Instead, the mechanism of bone resorption, as related to the parathyroid hormone, will be discussed in some detail, for this problem is now receiving more of the attention it deserves. It is being approached from two aspects: (1) the dissolution of the bone salt, and (2) the dissolution of the organic matrix of bone. The former approach has been chiefly concerned with the relationship between bone and citric acid; the latter has centred round the relationship between bone and mucopolysaccharides. The major component of the matrix, collagen, has been unduly neglected by those interested in the way in which bone is resorbed.

1. Bone Resorption: Parathyroid Hormone and Citric Acid

One of the more attractive concepts of the part played by the parathyroids in the resorption of bone is that the hormone causes the accumulation of citric acid which, in its turn, effects resorption of bone salt by means of its well-known chemical characteristics. The citric acid thus liberated is carried by the blood to the kidneys, where it is disposed of by metabolism rather than by excretion (Neuman and Neuman, 1958). This concept is based on the following observations. Citric acid is a normal constituent of bone and occurs to the extent of about 3 mM/100 g dry weight of bone (Dickens, 1941; Class and Smith, 1943). Some of the enzymes of the citric acid cycle have been shown to be present in bone (Dixon and Perkins, 1952; Perkins and Dixon, 1953; Laskin and Engel, 1956; Lees and Kuyper, 1957; Van Reen and Losee, 1958). Serum levels of citric acid and calcium respond in a parallel fashion to parathyroidectomy and parathyroid extract administration (Alwall, 1944; Freeman and Chang, 1950; L'Heureux and Roth, 1953; Elliott and Freeman, 1956*a*, *b*; Firschein *et al.*, 1958). Increasing the level of serum citric acid in intact animals, either by the administration of the acid itself (Chang and Freeman, 1950) or by its endogenous accumulation following injection of fluoroacetate (Freeman and Elliott, 1956), results in an elevation in the level of serum calcium. It is probable that in the intact animal such an effect of citric acid is partly due to indirect stimulation of the parathyroids secondary to a reduction in calcium ion concentration of the plasma. However, there is good evidence that citric acid can mobilize calcium independently of the parathyroids. Fluoroacetate injection will produce an elevation of plasma calcium even in the parathyroidectomized rat (Freeman and Elliott, 1956). Although Herndon and Freeman (1958) were unable to demonstrate a hypercalcemic response, in parathyroidectomized dogs, to infusions of citric acid, Freeman and Chang (1950) were able to show a slight response in the nephrectomized-parathyroidectomized dog. In addition, Elliott and Talmage (1957, 1958) found it possible to mobilize calcium by means of citric acid in the absence of the parathyroids. Using the technique of peritoneal lavage, in rats which had been nephrectomized and parathyroidectomized, these authors found that calcium could be mobilized by citric acid incorporated in the rinsing fluid.

It is pertinent to mention that vitamin D causes parallel rises in serum calcium and citric acid (Freeman and Chang, 1950; Harrison and Harrison, 1952; Harrison, Harrison and Park, 1958; Steenbock and Bellin, 1953). That the actions of parathyroid hormone and vitamin D on bone are not unrelated is evident from the important observation (Harrison *et al.*, 1958) that in the vitamin-D-deficient rat the hormone is ineffective in mobilizing calcium. Citric

acid has been further implicated in its relation to vitamin-D action by the work of DeLuca and his associates, who have neatly demonstrated that the vitamin reduces the ability of mitochondria of rat kidney (but not liver) to oxidize citric acid, whether the vitamin is administered *in vivo*, prior to the removal of the kidney (DeLuca, Gran, Steenbock and Reiser, 1957), or added *in vitro* (DeLuca and Steenbock, 1957). In a subsequent note (DeLuca, Reiser and Steenbock, 1959) it is suggested that vitamin D acts in this way by changing the mitochondrial structure and permeability.

If citric acid is to be implicated in a role of facilitating bone resorption one important question has to be answered: is there any direct evidence to support the idea that bone can produce and accumulate citric acid under certain cirumstances? Much indirect evidence exists such as was briefly summarized above, and indeed the notion has been previously suggested by several authors (Dixon and Perkins, 1952; Carlsson and Hollunger, 1954; Neuman and Neuman, 1958). Direct evidence is now available in the work of Kenny, Draskóczy and Goldhaber (1959) who have shown that, in tissue culture, resorbing bone is capable of metabolically producing and accumulating citric acid. This effect was engendered, in 2-day old mouse calvaria, by hyperoxia alone. Gaillard (1957) has made the important observation that parathyroid extract, when added to tissue cultures of the parietal bone of mouse embryos, will cause resorption of the bone. Unfortunately no biochemical data were recorded. Interpretation of this observation must remain uncertain for reasons which have been discussed in the "Introduction."

The time is ripe for intensive study of the biochemical mechanism of action of parathyroid hormone. Recent studies have indicated the presence in rabbit bone of the citric acid cycle enzymes, citrogenase, aconitase, isocitric dehydrogenase (Dixon and Perkins, 1952; Van Reen and Losee, 1958), and succinic dehydrogenase (Laskin and Engel, 1956). Little has been done to relate these enzymes to parathyroid action. *In vivo* manipulation of parathyroid function has been shown to affect the oxygen uptake, and citrogenase activity of bone when studied *in vitro*. Parathyroidectomy in rats was found to result in a lowered citrogenase activity in the epiphyseal and metaphyseal regions of the tibia and femur four to six weeks after the operation; no effect was seen after only four days (Perkins and Dixon, 1953). Administration of parathyroid extract to rabbits produced a diminished respiration in metaphyseal bone slices, but had no effect on anaerobic glycolysis (Laskin and Engel, 1956).

In focusing attention on the biochemical aspects of bone resorption it is important not to exclude from view the anatomical details. A tonic for those who err in this respect is the electron microscopic demonstration by Cameron and Robinson (1958) of the existence of crystals within the cytoplasm of osteoclasts but not in that of osteoblasts. Such evidence supports the idea of a phagocytic role for the osteoclast, a role discounted by McLean (1956). It is conceivable, of course, that the phagocytic osteoclast, as such, plays no primary role in bone resorption except to phagocytize incompletely-dissolved debris resulting from bone resorption produced by the biochemical activity of nonphagocytic bone cells.

2. Bone Resorption: Parathyroid Hormone and Bone Matrix*

An alternative mechanism of hormone action is that the hormone is primarily responsible for the mobilization of matrix, bone salt dissolution being of a secondary nature. Although this viewpoint should be given equal consideration it is experimentally less attractive. We know less about the biochemistry of the matrix than we do about the bone salt. Shetlar and Engel and their respective associates have been the most active in exploring this approach. Engel first showed that injection of parathyroid extract in rats at doses ranging from 10 to 1,600 units resulted in a rise in levels of mucoprotein† in the serum (Engel,

* The term matrix, as used here, covers all the organic structure of bone, including both collagen and the mucopolysaccharides of the ground substance.

† Much confusion exists in the terminology of these polymers. This is a confusion inherent in any rapidly developing field in which new components are being recognized and separated. The classification of Winzler (1958) may be used as a basis:

Mucopolysaccharides: high molecular weight polysaccharides containing hexosamine.
- *Neutral mucopolysaccharides:* contain hexosamine and neutral monosaccharides.
- *Acid mucopolysaccharides:* contain hexosamine, uronic acid and/or sulphuric acid.

Mucoproteins. protein combined with acid mucopolysaccharides in polar or other easily split types of linkage
Glycoproteins have the properties of proteins and contain 0 5 per cent or more of hexosamine firmly bound to protein.

The "seromucoid" fraction is synonymous with the heterogeneous fraction originally designated "mucoprotein" (Winzler, 1955). It represents the perchloric acid soluble, tungstate insoluble fraction, and, in terms of hexose content, accounts for about 10 per cent of the total protein-bound hexose of normal serum. The major component of the seromucoid fraction is α_1-glycoprotein (orosomucoid) in which the carbohydrate (40 per cent) is very firmly bound to the peptide moiety (60 per cent).

1952), and in the urine (Engel and Catchpole, 1953). These effects were associated with changes in the bone, which were interpreted as being a depolymerization and a solution of the glycoprotein ground substance. The effect of parathyroid extract (200 units IV) on the serum mucoproteins has been confirmed in humans (Kushner *et al.*, 1956). Shetlar (1956) has analysed these effects further, and found that administration of parathyroid extract to rats resulted in parallel rises in the levels of calcium, glycoprotein† and seromucoid† in the serum. However, parathyroid extract inactivated with formaldehyde produced no effect on serum calcium or glycoprotein, but still increased the level of seromucoid. Extract digested with pepsin had no effect at all. Perhaps the effect on the seromucoid is artifactitious. Contrary to the finding of Stewart and Bowen (1952), Kenny and Munson (quoted in Greep and Kenny, 1955) found that inactivation of parathyroid extract with formaldehyde destroyed both the calcium-mobilizing and phosphaturic activities of the extract. In any case, the effect of formaldehyde-inactivated extract on seromucoid must have had no relation to bone resorption as it was unaccompanied by a rise in serum calcium. This would seemingly make the effect of the active extract on serum glycoprotein more exciting, as this effect does appear to be related to the activity of the hormone. However, a disturbing facet of the data of Shetlar *et al.* (1956) is that 300 units of parathyroid extract, in contrast to 600 units, did not raise the glycoprotein but, if anything, lowered it, in spite of a concomitant rise in serum calcium. Similarly, in a subsequent paper, the intial response of the serum glycoprotein level to parathyroid extract administration appeared to be, if anything, a fall (Shetlar, 1958). Unfortunately none of these studies in animals included the effects of parathyroidectomy; it would be interesting to know whether or not effects opposite to those seen after extract administration would be observed following parathyroidectomy. This would facilitate the separation of the hormonal from the artifactitious responses to the extract. The work discussed above has not convinced the reviewer that these materials, whether they be serum glycoprotein or seromucoid, are coming from the ground substance of bone as a result of parathyroid hormone action.

Since the content of mucopolysaccharide material is low in bone matrix (less than 5 per cent of the dry, fat-free, organic matter) it is surprising that, in trying to demonstrate an effect of parathyroid hormone on the dissolution of the matrix, these materials should be chosen for analysis in blood and urine in preference to collagen. The latter substance, which constitutes 95 per cent of the dry, fat-free, organic matter, has been unnecessarily neglected. Investigation of the relationship between parathyroid hormone action and the collagen of bone matrix should prove fruitful. Electron microscopy has revealed the intimate relation between collagen fibres and the bone salt crystals (Robinson and Watson, 1955; Sheldon and Robinson, 1957). Collagen can be determined readily by analysis for one of its constituent amino acids, hydroxyproline, by the relatively specific method of Neuman and Logan (1950). Kenny, Draskóczy and Goldhaber (unpublished) attempted to determine whether or not the hydroxyproline content of the hydrolized media had increased as a result of bone resorption occurring in the tissue culture system of Goldhaber (1958). However, technical difficulties were encountered; there was found present in the medium a material which interfered with the hydroxyproline determination.

It is not uncommon that a study of a physiological defect in a mutant strain has led to a clearer understanding of the normal physiology of the wild strain. Such a defect exists in bone resorption in the *i.a.* (incisorless animals) mutant strain of rats discovered by Greep (1941). Schour and his colleagues (Schour, Massler and Greep, 1944; Schour *et al.*, 1949; Bhaskar *et al.*, 1950; Bhaskar, 1953; Mohammed, 1957) have unfolded the major anatomical defects to be found in the *i.a.* mutant strain by showing that these abnormalities, which are inherited by the *i.a.* strain as a simple Mendelian recessive factor, appear to be due to a generalized defect in bone resorption. Although Bhaskar *et al.* (1952) had reported that administration of high doses of parathyroid extract at an early and opportune time had shown a corrective effect on these abnormalities in the *i.a.* rat, the studies of Kenny, Toepel and Schour (1958) revealed that the defect was not due either to the existence of a chronic hypoparathyroidism or to a resistance of the *i.a.* rat bone to resorption, as measured by the hypercalcemic response, by parathyroid hormone. The physiological defect in bone resorption in the *i.a.* rat still remains to be elucidated.

3. Distribution of Calcium in Serum

The total calcium in the serum consists of three major components: the non-diffusible protein-bound fraction, the diffusible ionic fraction and the diffusible complexes. The approximate distribution of these fractions is given in Table 7.1, where it can be seen that the ionic fraction is the major component of the diffusible fraction. It is partly for this reason, but mainly for the reason that the ionic calcium can be determined only by the tedious frog heart method of McLean and Hastings (1935), that the diffusible fraction is frequently taken as representing the ionic fraction. The anions responsible for the small amount

† See footnote on previous page.

TABLE 7.1

Distribution of Calcium in Human Serum

Calcium fraction	Concentration	
	Neuman and Neuman (1958) mg/100 ml	Fanconi and Rose (1958) mg/100 ml
Non-diffusible		
1. Protein-bound*	3·3	3·3 (3·0–3·6)
Diffusible	6·5	6·2 (5·9–6·4)
2. Ionic	5·3	5·9 (5·6–6·2)
3. Complexed†	1·2	0·3 (0·1–0·4)
Total	10·0	9·5 (9·2–9·6)

* Mainly to albumin and α-globulin in the rat (Manunta, Saroff and Turner, 1957).

† Mainly with bicarbonate, phosphate and citrate (Neuman and Neuman, 1958).

of calcium in the form of complexes are assumed to be bicarbonate, phosphate and citrate. Other aspects of the state of calcium in the serum, in particular the question of the solubility of the calcium phosphate present, are critically discussed by Neuman and Neuman (1958).

The classical relationship between the concentration of ionized calcium and that of protein in serum, as represented by the equation of McLean and Hastings (1935), $(Ca^{++}) \times (Prot^{-})/(CaProt) = K = 10^{-2.22\pm0.07}$ (temperature 25°C, pH 7·35), is not completely reliable under all conditions, according to some investigators (Ludewig, Chanutin and Masket, 1942; Sartori, 1955; Lloyd and Rose, 1958). Others, however, are not dissatisfied with the relationship (Terepka, Toribara and Dewey, 1958; Hopkins, Connor and Howard, 1953) and, as a consequence, have suggested that measurement of the concentration of ionized or ultrafiltrable calcium is of no greater diagnostic value in cases of hyperparathyroidism than the simpler determination of the total calcium level. Lloyd and Rose (1958) disagree with this contention as they have shown that in 16 of 17 cases of hyperparathyroidism not only were the absolute values of ionized calcium increased but the percentage of the total calcium which was ionized was also increased. Parathyroidectomy in these patients resulted in a fall in the ionized fraction which was greater than that in the protein-bound fraction, which in 3 cases actually rose. They are forced to the inference that parathyroid hormone decreases the affinity of plasma proteins for calcium. The limits of plasma ionized calcium and of plasma protein-bound calcium in 7 normal men were found to be 5·9–6·5 and 3·4–3·9 mg/100 ml plasma water respectively (Rose, 1957; Fanconi and Rose, 1958). In discussing the discrepancies between their work and that of others, Lloyd and Rose stressed the significance of technical factors, emphasizing the importance of ultrafiltering in the presence of CO_2 at a concentration equal to the actual alveolar CO_2 tension of the individual patient. If the findings of Lloyd and Rose are accepted, then the determination of the plasma ionized calcium could be of particular value in the diagnosis of certain cases of primary hyperparathyroidism, which would be missed if the diagnosis were based on the level of total calcium only. Lloyd and Rose found an elevated level of plasma ionized calcium (7·4 and 6·8 mg/100 ml plasma water) in two cases of primary hyperparathyroidism in which the total calciums appeared normal (10·4 and 10·1 mg/100 ml).

The protein-bound calcium is not bound equally to each of the various plasma proteins. In the normal rat, albumin and α-globulin are mainly responsible, whereas parathyroidectomy results in a decrease in the fraction bound to albumin, and an increase in that bound to α-globulin (Manunta, Saroff and Turner, 1957). After oestrogen treatment, the calcium is associated with the β- and γ-globulin fractions.

The hypercalcemia associated with laying hens and oestrogen-treated roosters has been the subject of a very interesting study by Urist, Schjeide and McLean (1958). Oestrogen treatment of roosters will raise the serum calcium from normal levels of 10 mg per cent to levels around 100 mg per cent 5 days after injection with 100 mg of oestrone. With the aid of ultracentrifugation the authors found that oestrogen treatment resulted in the appearance of a phosphoprotein complex, which occurs normally in the laying hen but remains undetected in the rooster. The components of this phosphoprotein complex, identified as X_1, a phosphoprotein, and X_2, a phospholipid lipoprotein, were largely responsible for the binding of the extra calcium present in the serum. In a serum with a calcium concentration of 100 mg per cent, 79 per cent of the calcium was bound to the X_1–X_2 complex, 10 per cent to β-lipoprotein, and 5 per cent to albumin. The ultrafiltrable calcium remained unchanged at 6 mg per cent. Thus, oestrogens in birds are unable to influence the diffusible or ionized calcium, which is subject to parathyroid control. Polin and Sturkie (1958) found that the diffusible calcium level could be decreased in cocks or capons by parathyroidectomy, observing a fall of around 1·8 mg per cent 18–24 hours after the operation. A parallel fall in non-diffusible calcium occurred, as

would be expected from a normal operation of the McLean–Hastings relationship. The extreme variability of the response was attributed to the presence of accessory parathyroid tissue. This fact made it difficult to demonstrate that in the absence of parathyroid tissue oestrogen is incapable of evoking a hypercalcemic response. However, it was clear that in those birds which remained functionally parathyroidectomized, oestrogen treatment did not result in a rise in either the level of total or of non-diffusible calcium in the serum. The very interesting experiment which remains to be done is to see whether or not the phosphoprotein (X_1–X_4) complex described by Urist *et al.* (1958) would appear as a result of oestrogen treatment of the parathyroidectomized rooster.

The observation first made over 35 years ago (Bogert and Plass, 1923) that the serum calcium of the foetus is 1–2 mg per cent higher than that of the mother has yet to be satisfactorily explained. In discussions of this fact (Bodansky and Duff, 1939; Greep, 1948; Smith, 1951) two earlier papers, the one by Nicholas, Johnson and Johnston (1934) and the other by Andersch and Oberst (1936), have been largely ignored, no doubt partly because these authors failed to emphasize certain aspects of their findings. The data of Andersch and Oberst revealed that, in spite of the higher total calcium in cord serum (mean 11·8 mg per cent) relative to maternal serum (mean 10·4 mg per cent), the diffusible calcium levels were equal (5·3 and 5·5 mg per cent respectively). Thus, if the diffusible calcium levels are similar one does not have to invoke a system for active transport of calcium across the placental barrier, as suggested by Smith (1951). The question narrows down to what causes the greater level of non-diffusible calcium in foetal blood. It is not due to an increased total protein concentration, as the protein in foetal blood is lower (Andersch and Oberst, 1936; Widdowson and McCance, 1956). It would appear then that the higher foetal calcium must be due to an increased calcium-binding power by one or more of the colloids in the foetal plasma, as was first suggested by Duckworth (1942). It is fascinating to speculate that perhaps the increase in non-diffusible calcium concentration in foetal blood is due to the production, under oestrogen control, of a phosphoprotein complex similar to that described by Urist *et al.* (1958) in birds. The foetus, which in other aspects appears to possess several phylogenic remnants, may retain this ability to respond to oestrogen by production of a phosphoprotein complex, as is found in frogs and fish (quoted in Urist *et al.*, 1958) as well as in birds. An observation which is of particular relevance to this subject is that of Hallman and Salmi (1953) who found that the serum entering the human infant from the placenta (umbilical vein) contained more calcium (0·3–2·3 mg per cent) than the serum returning to the placenta (umbilical artery) Even the serum from the umbilical artery had, in general, higher levels of calcium than maternal venous blood. It would appear, then, that whatever is responsible for the maintenance of a high non-diffusible calcium level in the foetus is located in the foetal side of the placenta. The finding by Economou-Mavrou and McCance (1958) that, in the pig, the foetal serum from mixed arteriovenous blood contains a much higher level of calcium (17·6 mg per cent) than the maternal blood (11·2 mg per cent) is interesting. This difference of over 6 mg per cent would make this animal particularly suitable for studying this problem in mammals. It would be intriguing to know if this large difference was due entirely to the non-diffusible fraction and, if so, whether or not this increment was under oestrogen control as it is in the adult bird. It is of course well known that oestrogen levels in the maternal blood increase throughout pregnancy, reaching values, at term, which are ten times those of normal.

The distribution of calcium in the brain is strange, for Streicher (1958) has found a bilateral asymmetry in the concentration of calcium in rat brain. He was able to demonstrate differences between the two halves of rat brain at all ages from 1 to 12 months; some values differed from each other by over threefold. He suggested that the calcium may be complexed as phosphate in the non-parenchymal tissue such as blood vessels, assuming that the difference cannot be due to a difference in the ionic fraction.

4. Parathyroid-Serum Calcium Relationship

There is no need to discuss fully the evidence which supports the idea that the serum level of ionized calcium, and perhaps of inorganic phosphate, controls the secretory activity of the parathyroid glands, for this has recently been adequately reviewed (Greep, 1948; Greep and Kenny, 1955). An observation by Kenny (1957) that inorganic phosphate when mixed with parathyroid extract suppressed the calcium-mobilizing activity of the latter, in the parathyroidectomized rat, may be relevant. This effect was not due to any direct effect of the injected phosphate on the serum calcium level, for injection of the extract and phosphate simultaneously, but at separate sites, resulted in no suppressing action. The effect was reversible, in that removal of the phosphate from the extract, by dialysis, restored the activity to normal. It thus appears as if inactivation occurred by a chemical interaction between the hormone and phosphate. The question whether or not inorganic phosphate added to parathyroid extract would have any influence on the phosphaturic activity of the

latter has not been investigated. Although the quantity of phosphate required to produce this effect was high in terms of concentration in the extract (4 mg P/ml), the injection of phosphate had no effect on the level of serum phosphate 6 hours later. It is not inconceivable that high physiological levels of phosphate might be responsible for inactivating the hormone, particularly at its site of action, bone, where, as a result of the action of the hormone, local concentrations of both calcium and phosphate would undoubtedly increase. Whereas the level of calcium ions in the serum is responsible for controlling the secretion of the hormone from the gland, perhaps the level of inorganic phosphate may be accountable for regulating the activity of the hormone once secreted. Such an auto-regulatory mechanism would be consistent with the precise homeostasis which exists for the calcium of the serum.

5. Intestinal Absorption of Calcium

It has long been assumed that the parathyroid glands have no direct control over calcium absorption from the gastro-intestinal tract. Categorical statements to this effect are made in the review literature, with little evidence to support them (Nicolaysen, Eeg-Larsen and Malm, 1953; Irving, 1957). The last-named cites no references, whereas Nicolaysen *et al.* (1953) mention four papers in connexion with this aspect of calcium metabolism, Albright and Reifenstein (1948), Robertson (1942), Bülbring (1931), and Carlsson (1951); unfortunately the two last named are misquoted. Bulbring (1931) made no claim to have demonstrated no effect of parathyroid hormone on calcium absorption, as was accredited to her by Nicolaysen *et al.* She did show that in the rat, parathyroidectomy resulted in increased retention of calcium, an effect which could be reversed by the administration of parathyroid extract. These effects, of course, were probably due to the well-known effects of the hormone on calcium excretion in the urine. Although Albright and Reifenstein (1948) are quoted in support of the contention that the parathyroids exert no control over calcium absorption, it is difficult to find any clear-cut evidence in their monograph. In one place (p. 134) the hormone is listed as causing a slight increase in absorption; otherwise the authors quote an earlier paper (Albright, Bauer, Ropes and Aub, 1929) in which the effect of parathyroid extract in normal men was reported. In this study no consistent effects on the calcium content of the faeces as a result of parathyroid extract injection was observed. However, conclusions based only on observations of effects on faecal calcium are somewhat precarious, due to the fact that the faecal calcium consists of two components, endogenous and unadsorbed calcium.

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For, in rats, Carlsson (1951) has shown that parental administration of calcium lactate increased (not "reduced" as quoted by Nicolaysen *et al.*, 1953) the absorption of Ca^{45} from the intestine, an effect attributed by Carlsson to a non-specific inhibition of gastro-intestinal motility. Yet, to be reconciled with the findings of Albright *et al.* (1929) is the familiar observation, reviewed by Robertson (1942), that faecal calcium excretion is considerably reduced in hyperparathyroidism, indicating a possibly increased absorption. Again, this may well be due to an inhibition of gastro-intestinal motility secondary to the accompanying hypercalcemia.

The ideal experimental situation would be to study the actual absorption of calcium from the intestine. This approach has been used by Talmage and Elliott (1958), who measured the rate of radiocalcium disappearance from ligated sections of rat intestine. The rate of disappearance was decreased by 50 per cent 2 to 4 hours following parathyroidectomy. It would be interesting to know if the hypocalcemia *per se* was responsible for this effect. In sharp contrast to these findings, Sekckawa (1956) reported that parathyroidectomy augmented calcium absorption from the intestine.

In view of these conflicting observations and lack of solid evidence the author of this review is not convinced that the question, whether or not parathyroid hormone has any direct action on calcium absorption from the intestine, has been settled. It may, of course, have indirect effects secondary to the level of ionized calcium in the plasma, a factor known to affect smooth muscle motility and, perhaps as a result absorption, from the intestine in general. Consequently, in studies such as the preliminary one reported by Talmage and Elliott (1958), it would be necessary to control or eliminate the effects of the concurrent hypocalcemia either by maintaining the plasma calcium at a normal level by continuous infusion or by controlling intestinal motility pharmacologically. It would also be interesting to see whether or not the decreased absorption of calcium seen after parathyroidectomy was specific for calcium or of a general applicability to other materials such as glucose, whose rate of absorption would presumably be sensitive to non-specific effects such as changes in motility.

It is certainly an area which requires further investigation. It is not a simple problem from the point of view of experimental approach, but the latter is made easier by the availability of radio-isotopes. The everted gut-sac preparation of the small intestine should be applicable to this problem. This technique, described earlier by Wilson and Wiseman (1954), has been successfully applied by Schachter and Rosen (1959) to an *in vitro* study of the mechanism of active trans-

port of Ca^{45} in relation by its dependence on vitamin D and oxidative metabolism.

6. Renal Excretion of Calcium

There is no disagreement over the fact that, in the dog, over 99 per cent of the filtered calcium is reabsorbed by the renal tubule (Jahan and Pitts, 1948; Chen and Neuman, 1955*a*; Poulos, 1957). Both Chen and Neuman and Poulos were unable to demonstrate a tubular reabsorptive maximum (T_m) for calcium, even when the plasma calcium was raised from 8·3 to 37 mg per cent by infusion of calcium. The small amount of filtered calcium which is excreted in the urine is thought to be in an un-ionized form, perhaps partly as a complex with citrate (Chen and Neuman, 1955*a*). The fact that calcium reabsorption was shown to be independent of water reabsorption (Chen and Neuman, 1955*a*) and that metabolic inhibitors such as phlorizin, dinitrophenol and sodium azide depressed calcium reabsorption, leaving sodium and potassium reabsorption unaffected (Chen and Neuman, 1955*b*), indicates that calcium reabsorption is an active process.

A matter which is unsettled is whether the parathyroids have any direct control over the renal handling of calcium, or whether parathyroid effects on urine calcium are only reflections of the level of ultrafiltrable calcium in the plasma, or, more specifically, the filtered load. The work of Jahan and Pitts (1948), in dogs, remains the only thorough study of this problem. They attributed the rise in urine calcium, seen over 14 hours after subcutaneous injection of parathyroid extract in dogs, to an increase in filtered calcium as a result of the rise in plasma ultrafiltrable calcium, and concluded that "parathormone does not interfere with calcium reabsorption by the renal tubules." The present author questions this interpretation of the data, holding the opinion that the important factor contributing to the hypercalciuria is not the increased filtered load but is a decreased TR/GF ratio*. Although the total quantity of calcium reabsorbed was increased following parathyroid extract administration, the percentage of the filtered calcium which was reabsorbed was significantly ($p < 0·001$) reduced from an average of 99·7 per cent in the normal dogs to a mean of 93·5 per cent in the dogs treated with hormone (Table 7.2). It is unlikely that

* TR=tubular reabsorbate; GF=glomerular filtrate. The ratio may be expressed either as a fraction (TR/GF) or as a percentage (TR×100/GF). Changes in this ratio imply, of course, only a net effect on reabsorption. They do not rule out the possibility of tubular secretion being responsible for this net effect. However, this possibility does not render the filtered load of any greater importance.

TABLE 7.2

Renal Excretion of Calcium in the Dog and its Modification by Parathyroid Hormone and by Calcium Infusion

(*From* Jahan and Pitts, 1948; *and* Poulos, 1957.)

	Glomular filtration rate (cc/min)	Urine flow (cc/min)	Plasma or Serum Ca: Total (mg %)	Plasma or Serum Ca: Ultrafiltrable (mg %)	Renal calcium: Filtered (GF) (mg/min)	Renal calcium: Excreted (mg/min)	Renal calcium: Reabsorbed: Total (TR) (mg/min)	Renal calcium: Reabsorbed: TR×100/GF (%)
Normal[1]	66·6	4·32	7·3	4·4	2·92	0 008	2·91	99·7*
Normal+ Parathyroid Extract[1]	65·8	4·02	11·9	6·6	4·35	0·282	4·07	93·5*
Normal+Ca Infusion[2]	56·5	1·64	11·0	6·3	3·56	0 025	3·54	99·4
	45·8	0·38	22·1	15·1	6·93	0 045	6·88	99·3
	57·4	3·30	28·2	20·0	11·49	0·72	10·77	93·7
	51·4	6·53	36·7	26·4	13·58	2·26	11·32	83·4

* The difference between these means is significant ($p<0·001$).

NOTES.

1. *From* Jahan and Pitts (1948). Each value represents the mean of a total of six experiments in two dogs. The hormone-treated dogs received two doses of 300 units each of parathyroid extract subcutaneously at 20 and 14 hours before the start of the experiment. Note the unusually low level of normal serum calcium, 7·3 mg %.

2. *From* Poulos (1957). Each value represents the mean of duplicate experiments in a single dog. The dog in the last three experiments received a continuous infusion of calcium chloride in order to raise the level of serum calcium above normal.

this decrease in the percentage reabsorbed was due to increased filtered load *per se*, as Poulos (1957) found that increasing the ultrafiltrable calcium concentration in the plasma, by infusion of calcium from a normal value of 6·3 mg per cent to 15·1 mg per cent, had no effect on the percentage reabsorbed (TR × 100/GF), the value not falling below 99·3 per cent (*see* Table 7.2). As a consequence, increasing the filtered load by over twofold had negligible effects on the quantity of calcium excreted; it raised the urine calcium from 0·025 to 0·045 mg/min, certainly not the thirty-five fold increase (from 0·008 to 0·282 mg/min) observed by Jahan and Pitts after parathyroid extract administration (Table 7.2). The maximum concentration of ultrafiltrable calcium obtained by the latter authors was 7·0 mg per cent, well within the range found by Poulos to have negligible effects on calcium excretion. The work of Poulos is very important, for it emphasizes the fact that marked changes in the concentration of ultrafiltrable calcium in the plasma, and hence, changes in the filtered load of calcium, have little effect on calcium excretion, and thus may be largely ignored relative to changes generated by other mechanisms. Any important changes in calcium excretion will have to be brought about by changes in the TR/GF ratio.

The other notable studies in this area have been conducted in the rat by Talmage and his associates. They very convincingly draw attention to the fact that, in the rat, parathyroidectomy is followed immediately, within 2 to 4 hours, by a sharp rise of over sixfold in the excretion of calcium in the urine. This rise occurs in the face of a falling level of serum calcium (Talmage and Kraintz, 1954; Talmage, Kraintz and Buchanan, 1955; Talmage, 1956). Data culled from these papers are plotted in Fig. 7.1. No further analysis of the renal mechanism was undertaken, but it is difficult under the circumstances to avoid the conclusion that parathyroidectomy resulted in a decreased tubular reabsorption of calcium which dwarfed the decreased excretion resulting from the probable decrease in filtered load. We are thus confronted with an apparent paradox. The data of Jahan and Pitts indicate, at least to the reviewer, that parathyroid extract (and presumably the hormone) reduces the TR/GF ratio in the dog; whereas the work of Talmage and his associates implies that, in the rat, parathyroidectomy also reduces the TR/GF ratio. The latter conclusion is, of course, very tenuous as it is based not on a critical analysis of renal function but on grosser data. Many differences exist between the two types of experiments. There is the dissimilarity of species between the rat and the dog; Jahan and Pitts did not study the immediate effects of parathyroid extract administration; one group studied the effects of parathyroidectomy, the other the effects of parathyroid extract administration. Thus, until these experiments are confirmed and extended it is not

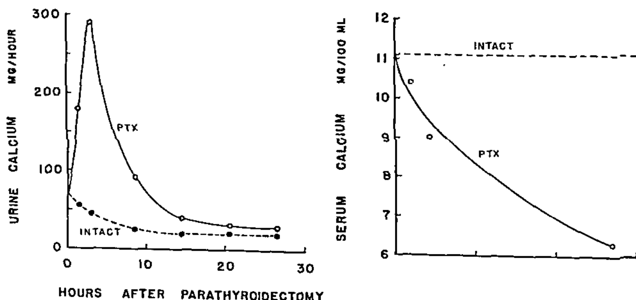


FIG. 7.1. RENAL EXCRETION OF CALCIUM AND ITS MODIFICATION BY PARATHYROIDECTOMY IN RATS

Note the greater than sixfold increase in calcium in the urine of the parathyroidectomized (PTX) rats within 4 hours, in spite of a concurrent fall in the level of serum calcium. From *Talmage and Kraintz* (1954); *Talmage, Kraintz and Buchanan* (1955); and *Talmage* (1956).

possible to come to any definite conclusion concerning the nature of the physiological control of the renal excretion of calcium exerted by the parathyroids.

Though a discussion of the relationship between the parathyroids and the renal handling of phosphorus is not entirely relevant to the topic under consideration, the present author cannot resist the opportunity of stating his belief in the hormonal nature of the phosphaturic activity exhibited by the parathyroids. The one paper which apparently tests one's faith in this concept is that by Stewart and Bowen (1952). Though the findings of these authors may be true in the dog, they do not hold for the rat. Kenny and Munson (1959) have been unable to confirm in the rat some of Stewart and Bowen's observations which were made in the dog. First, parathyroid extract, inactivated by formaldehyde, lost both calcium-mobilizing and phosphaturic activity to approximately the same extent; secondly, spleen extract, prepared by the method used for the preparation of parathyroid extracts, possessed no detectable phosphaturic or calcium-mobilizing activity (Kenny and Munson, 1959). In addition, at least one purified preparation of parathyroid hormone has been reported to exhibit both activities in the rat (Friedman and Munson, 1958*b*). To this evidence must be added the thoroughly documented observation, known since 1911, the year of its original discovery by Greenwald (1911) in the dog, that parathyroidectomy in several species results in a decreased excretion of phosphorus in the urine. Part of the phosphaturic activity of parathyroid extract may be artifactitious, but most of it is hormonal in origin. The apparent species difference between the dog and the rat, so far as phosphaturic responses are concerned, is not the only one that has been observed. Triiodothyronine has been reported to produce a phosphaturia in dogs regardless of whether the dogs were intact or parathyroidectomized (Beisel *et al.*, 1958). In rats, the same hormone at a dose of 10 mg/kg, 100 times the dose used by Beisel *et al.*, failed to evoke any phosphaturia (Kenny, unpublished observations).

III. NUTRITIONAL ASPECTS OF CALCIUM METABOLISM

The direct relationship, or lack of it, between the parathyroid glands and calcium absorption can be covered in few words. However, it is felt that the subject of calcium absorption and retention is so basic to an understanding of calcium nutrition that recent developments in this area, whether or not the parathyroids are directly or indirectly involved, will be discussed at some length.

A. Dietary Factors and Calcium Absorption

This is not the place to discuss the dietary factors known to affect calcium absorption, including those which enhance calcium absorption such as vitamin D, amino acids, lactose, and those which hamper absorption such as phosphates, phytates, oxalate and fatty acids. The influence of these factors is so well covered in the excellent monograph by Irving (1957) that little additional mention will be made of them in this review.

Lactose and the amino acids, L-lysine and L-arginine, have been shown to increase the absorption of calcium in the normal and vitamin-D-deficient rat (Wasserman, Comar and Nold, 1956; Wasserman *et al.*, 1957; Lengemann, Comar and Wasserman, 1957). The mechanism of these effects is unknown (Wasserman *et al.*, 1956). These authors measured calcium absorption by administering radiocalcium plus carrier calcium by stomach tube and determining the radiocalcium present in the faeces and gastro-intestinal contents as well as the radiocalcium uptake by the femur. They point out that, although vitamin D was less effective than L-lysine in stimulating the absorption of calcium from the gut, it caused equal deposition in the femur. This was interpreted as meaning that, over and above the well-known action of vitamin D on intestinal absorption, the vitamin has other actions on bone mineralization or kidney excretion or both. The authors point out the pitfall one might encounter if one uses bone uptake as the only indicator of calcium absorption.

B. Adaptation

At the heart of all studies on calcium metabolism has been the balance experiment, in which the calcium intake and the total excretion of calcium are measured and compared. As our knowledge of calcium metabolism gradually increased, interpretation of the balance experiment became more complicated. Such factors as the endogenous loss of calcium through intestinal secretion, and adaptation to low intakes of calcium, have made extrapolation of the results more precarious. The balance experiment, used wisely, is still an extremely important tool, but used otherwise it may be effective for one purpose only, as an indicator of previous dietary intake.

Nicolaysen, Eeg-Larsen and Malm (1953) have re-emphasized the ability of man and animals to adapt to low levels of calcium intake by increased efficiency of absorption, proposing the intermediary of an "endogenous factor" (Nicolaysen, 1943). It was

suggested that the endogenous factor linked the body stores of calcium, on the one hand, and the efficiency of calcium absorption as influenced by vitamin D, on the other hand, and that vitamin D was necessary for its action (Nicolaysen, 1943). That the organism can adapt to lowered calcium intakes and, by more efficient absorption, remain in positive balance, is now well-documented in rats (Henry and Kon, 1953; Rottensten, 1938; Nicolaysen, 1943; Hansard and Plumlee, 1954), in dogs (Gershoff, Legg and Hegsted, 1958) and in humans (Walker, Fox and Irving, 1948; Nicolaysen *et al.*, 1953; Malm, 1958). In their studies of dogs which had adapted to low levels of calcium intake Gershoff *et al.* (1958) were unable to put dogs into negative balance, even with a diet containing only 0·034 per cent calcium, the lowest calcium diet which could be conveniently prepared. Nicolaysen (1956) found that rats would remain in satisfactory balance even on very low calcium intake (0·04 and 0·098 per cent) until death. In spite of all the evidence in favour of the theory of adaptation, McCance (1954) remains adamant in his refusal to accept it.

Although most investigators are agreed on the existence and importance of adaptation there is less concurrence with the idea of an endogenous factor, which survives to this day only as a concept with no evidence, or even suggestions, to associate it with any particular hormone or biochemical. Henry and Kon (1953) claimed that their data did not support the concept as proposed by Nicolaysen (1943). However, Henry and Kon did not do the particular experiment on which Nicolaysen based his idea. The latter investigator used rats which were maintained on diets containing either 0·95 or 0·2 per cent calcium. When the rats maintained on these diets, both of which were supplemented with vitamin D, were put on a 100 mg/day diet, the absorption of calcium was greater in the rats which had been maintained on the low calcium diet. However, when rats were kept on the 0·95 and 0·2 per cent calcium diets without vitamin D and then placed on a 75 mg/day diet, there was no significant difference in the absorption of calcium between the two groups of rats. Henry and Kon did not maintain their rats on any diet with a calcium concentration less than 0·73 per cent in their experiments with a vitamin-D-deficient diet. In other words, they did not test the ability of the rats to adapt (more efficient absorption) to a low calcium diet without vitamin D. They did, of course, show that rats could adapt to a low calcium diet in the presence of vitamin D. This author fails to understand the evidence claimed to support the idea of an endogenous factor in a subsequent paper by Haavaldsen, Mortensen and Nicolaysen (1956).

The fact that the organism can adapt to low levels of calcium intake does not mean that all organisms can adapt to any level, however low. Adaptation is not immediate, and during the initial period after the imposition of a diet lower in calcium a negative balance will probably exist. In the elegant studies of Walker *et al.* (1948), subjects who had been on a dietary calcium intake of about 1 g, when put on a daily intake of 0·5 g went into negative balance for 6 to 7 weeks, after which time they went into positive balance. Very little change in urinary excretion of calcium occurred over the experimental periods. In an extensive study of calcium requirements and adaptation in adult men, Malm (1958) classified adapttation types as follows: Type A, immediate adaptation, seen in 11·5 per cent of the 26 men studied; Type B, initial negative balance followed by adaptation, seen in 77 per cent of the subjects; Type C, no signs of adaptation, seen in 11·5 per cent of the subjects. The time for type B subjects to adapt from an intake of 940 mg to one of 460 mg ranged from 42 to 252 days. Malm also found that adaptation was accompanied by increased efficiency of absorption and decreased faecal excretion, which are of course related. There was no evidence of a reduction of urinary calcium being of any significance in the process of adaptation. It is obvious from the above discussion that the measurement of balance data, often done over a period of one or two weeks after imposition of a low calcium diet, with no regard for previous dietary history, leads to unreliable conclusions concerning calcium requirements of individuals.

In view of the recent suggestion of Talmage and Elliott (1958) that parathyroid hormone facilitates the absorption of calcium from the intestine, and of the observation of Harrison, Harrison and Park (1958) that the calcium-mobilizing activity of parathyroid hormone is ineffective in the absence of vitamin D, it would be tempting to speculate on the possibility of the parathyroids being responsible for the secretion of Nicolaysen's endogenous factor. It would be interesting to do the experiment of Talmage and Elliott in rats which had been maintained on diets with and without vitamin D. If Nicolaysen's endogenous factor is a reality, and if it is secreted by the parathyroids, then parathyroidectomy should have no effect on calcium absorption from the intestine in the vitamin-D-deficient rat.

C. Endogenous Loss of Calcium

The practicability of using radiocalcium in human studies has made it possible to sharpen our knowledge concerning that aspect of calcium metabolism known as the endogenous loss of calcium. The latter term includes the calcium which is excreted in the urine and the calcium of endogenous origin which appears,

unabsorbed, in the faeces. The finding of intravenously-administered radiocalcium in the faeces leaves no doubt as to the fact that endogenous calcium is secreted into the gastrointestinal tract and is excreted in the faeces (Bronner *et al.*, 1956). It is becoming clear that previous estimates of the endogenous loss of calcium through intestinal secretion were on the high side; earlier estimates ranged from 300 to 1,000 mg/day (Nicolaysen *et al.*, 1953; Logan, 1940). Recent estimates, based on studies with radiocalcium, are more conservative. Blau *et al.* (1957) computed the endogenous secretion into the gastrointestinal tract to be 150 and 290 mg/day in two patients whose endogenous faecal calcium levels were 85 and 99 mg/day respectively. Bronner and Harris (1956) found endogenous faecal calcium levels of 120 mg/day in an adult, and an average of 80 mg/day in 6 boys aged 11–16 years. However, in some individuals the endogenous secretion may far exceed these values, as Malm (1958) reported that the faecal calcium of one subject (subject No. 12, balance period No. 62) exceeded the calcium intake by more than 600 mg.

The endogenous loss of calcium in the normal individual remains fairly constant, increasing gradually with age both in humans (Knapp, 1947) and in rats (Henry and Kon, 1953). The urinary excretion of calcium is relatively independent of calcium intake. Malm (1958) was unable to demonstrate any significant difference between the mean amounts of urinary calcium excreted by 22 men at two levels of calcium intake. For intakes of 940 and 463 mg/day the urinary calcium values were 231 and 201 mg/day respectively. Similar findings had been reported previously by Walker *et al.* (1948) in a series of three subjects. These findings are supported by studies in rats (Henry and Kon, 1953). However, by changing the calcium intake over tenfold from 226 to 2,339 mg/day Knapp (1947) was able to exact a significant change in urine calcium excretion from 131 to 229 mg/day in 7 men. It is obvious that ordinarily changes in urinary excretion of calcium play no significant part in the process of adaptation. Calcium excretion by the kidney is of course subject to endocrine control by the parathyroids as is discussed elsewhere.

The mechanism of the regulation of the intestinal secretion of calcium is unknown. Earlier studies in dogs (Nicolaysen, 1934) and in man (McCance and Widdowson, 1939; Baylor *et al.*, 1950) had claimed that the secretion was not influenced by the level of plasma calcium. However, these conclusions were based on the observation that after administration of either calcium intravenously or parathyroid extract subcutaneously the faecal calcium was unchanged. The two factors contributing to the amount of faecal calcium, namely intestinal absorption and secretion, were not separated. Under these conditions interpretation is complicated, especially as a result of Carlsson's finding (1951) in the rat, that parenteral administration of calcium lactate increased absorption of ^{45}Ca from the intestine. Carlsson attributed this effect to a non-specific inhibition of gastro-intestinal motility. If this occurred in the dog and human studies mentioned above then, perhaps, injection of parenteral calcium did increase intestinal secretion, an event which may have been masked by a possible concomitant increase in absorption.

D. Normal Calcium Requirements

The daily allowance of calcium recommended by both the American and British authorities is 800 mg/day for adult men. What is the current thinking on the adequacy of this recommendation? The concept of what is required for the maintenance of body stores of calcium at physiological levels has undergone considerable change in recent years as a result of the evidence discussed above. In the United States, Hegsted has been active in emphasizing the necessity for a re-evaluation of our concept of minimum calcium requirements. He has stressed the importance of the adaptation theory by enunciating two biological laws: (1) that "the body makes more efficient utilization of a nutrient when the body is in need;" (2) that "all so-called normal adults whether they are found eating low calcium diets or high calcium diets are in balance" and show no signs of ill health attributable directly to the level of calcium intake (Hegsted, 1954).

Malm (1958) has compiled a survey of the literature concerned with the evaluation of minimum calcium requirements. It is obvious that the estimates differ widely, ranging from 200 to over 1,000 mg/day. Many of the studies reporting low requirements were done on individuals racially different from Caucasians, and located in tropical climates in which factors such as sweating and greater exposure to sunlight might play considerable, hence subversive, roles. An average of 419 mg/day was required by 5 young Chinese college women (Kung and Yeh, 1937); 5 East African Bantu boys, aged 15–17 years, required 430 mg/day (Henderson and Kelly, 1929–30); 3 Indian men required 383 mg/day (Basu, Basak and Rai Sircar, 1939); Hegsted, Moscoso and Collazos (1952) found that 10 Peruvian men required an average of 216 mg/day. Estimates of requirements for North Americans have usually been higher. Steggerda and Mitchell (1951) reported a requirement of 518 mg/day for 13 men; 136 women were found to require from 730 to 920 mg/day (Ohlson *et al.*, 1952); 124 young college women required 816 mg/day (McKay *et al.*, 1942); 9 women, all over 50 years of age, required 1,067 mg/

day (Roberts, Kerr and Ohlson, 1948). Some investigators feel that these high estimates indicate only one thing: that the previous dietary intake of calcium was high in these individuals. Malm (1958) concluded from his own data that the average calcium requirement of 26 Norwegian male prisoners, 20-69 years old, was 440 mg/day (range 337–890). His studies, which were carried out over extensive periods of time, included observations of the calcium balances of men on a daily intake of 459 mg/day for an average period of 240 days. Twenty-two of the 26 men had been studied for an average period of 218 days on an intake of 940 mg/day before being placed on the low calcium diet.

One factor which of necessity has had to be ignored experimentally, if not mentally, in all investigations of the human requirements for calcium is the endogenous loss of calcium in sweat. The dermal loss of calcium has been examined *per se* by Mitchell and Hamilton (1949) and by Johnston, McMillan and Evans (1950). The former investigators reported that in 6 men an average of 149 mg/day was lost in an environment at 29°C and at a relative humidity of 50–52 per cent and an average of 484 mg/day at 38°C and a relative humidity of 68 per cent. The latter found that, under conditions of profuse sweating, 4 young women lost an average of 8·5 mg/hr (range 4·2–14·3 mg/hr). Heavy losses such as reported in these papers would, of course, have a marked influence on the estimation of minimum requirements. This difficulty was discussed by Malm (1958), who felt that, in his study, the Norwegian environment was not conducive to excessive loss by this route. However, it may be of considerable significance in those studies conducted in hot climates. It would, of course, if ignored, render the estimate falsely low. The possibility of dermal loss of calcium has not received the attention it deserves in the interpretation of balance data based solely on urinary and faecal losses.

Even if the high estimates of requirements for North Americans are casually dismissed as being unreal and merely reflecting a previous high intake of calcium, it is necessary to explain the difference between the 440 mg/day requirement of Malm (1958) and the 216 mg/day value of Hegsted *et al.* (1952). Although considerations of sunlight, environmental temperature, and heredity may be freely invoked in explanation of the discrepancy, perhaps the most important factor is still differences in life-long dietary history. The men studied by Hegsted *et al.* (1952) had presumably been on very low intakes all their lives, whereas the Norwegian prisoners had undoubtedly been on diets containing much greater amounts of calcium. Even the regular prison diet contained not less than 650 mg/day. Malm made no attempt to determine minimal values; adaptation to calcium intakes below 459 mg/day was not investigated.

In estimating the calcium requirements a basic assumption is invariably made: that the requirement is *that* intake level which maintains a zero or slightly positive balance. However, no evidence exists for man to show how much under-saturation of the skeleton, or prolongation of a negative balance, is necessary to endanger the health and performance of the individual. It is assumed that maximum saturation of the skeleton is the teleological ideal. This assumption is challenged by Hegsted and his associates (Hegsted *et al.*, 1952; Hegsted, 1957; Gershoff *et al.*, 1958), who emphasize both the lack of evidence in its support, and the data of Henry and Kon (1953), which indicated that, in rats, calcium restriction appeared to deposit calcium in a more stable form.

IV. DEVIATIONS OF CALCIUM METABOLISM

Normal calcium metabolism may be disturbed as a result of many factors of physiological, pharmacological, pathological and nutritional origin. These include conditions such as pregnancy, lactation, vitamin-D deficiency states, hypervitaminosis D, and diseases of the parathyroid glands. Metabolic disorders related to vitamin D are outside the scope of this review. Diseases of the parathyroid glands, hyperparathyroidism and hypoparathyroidism, will be discussed with emphasis on the problem of diagnosis rather than that of treatment, about which there is little disagreement.

A. Calcium Metabolism in Pregnancy and Lactation

Little can be added to other reviews of calcium metabolism and the role of the parathyroids during pregnancy and lactation (Greep, 1948; Irving, 1957). It is clear that pregnancy places no great strain on the body stores of calcium, although it is evident that a mild case of hyperparathyroidism exists in many pregnancies (Greep, 1948). There is a tendency for the serum calcium level to fall during the later stages of pregnancy. Supplementing the diet with extra calcium or vitamin D does not have any effect on the serum calcium level in humans (Newman, 1953; Lapan and Friedman, 1958). Parathyroidectomized rats, which normally suffer no major inconvenience if maintained on an adequate diet, are adversely affected if allowed to become pregnant, particularly in the later phase, when tetany and death usually ensue at or about the time of the onset of labour (Bodansky and Duff, 1939, 1941).

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in urinary excretion of phosphorus. These responses may be explained on the basis of the resultant rise in serum calcium which, in normal persons, would tend to inhibit parathyroid secretion, leading to reduced excretion of phosphorus. In the hypoparathyroid patient there would be no secretion to inhibit, and the rise in serum phosphorus, which unexplainedly accompanies the infusion of calcium, would make itself felt in the form of increased filtered load, hence increased excretion of phosphorus. The response in the hyperparathyroid patient could conceivably be variable, depending on the degree with which the tumor responded to the inhibiting effect of the increased serum calcium. In general, these studies of Howard *et al.* (1953) have been confirmed by others (Goldman and Bassett, 1954; Justin-Besancon *et al.*, 1954; Kyle, Schaaf and Erdman, 1954; Nordin and Fraser, 1954, 1956; Chambers *et al.*, 1956; Thomas, Connor and Morgan, 1958). To be reconciled with these findings is the earlier observation by Albright and Sulkowitch (1938), that infusion of calcium in a patient with idiodathic hypoparathyroidism resulted in a decrease, not an increase, in excretion of phosphorus. Not all clinical investigators are satisfied with the calcium infusion test as a diagnostic aid. In particular, Goldzieher and his associates (Goldzieher, Heaney and Fairweather, 1957; Goldzieher, 1958) express the opinion that it "does not seem to be a clear-cut diagnostic aid." From the physiological point of view, perhaps the most interesting aspect of the calcium infusion test is the still unexplained origin of the phosphorus which accumulates in the serum. It is not due solely to retention of phosphorus by the kidney, for it accummulates in conditions where there is an increase in the urinary excretion of phosphorus. This problem deserves further study on its own merits.

3. Phosphorus Deprivation Test

Reifenstein (1958) formulated this procedure to facilitate the diagnosis of hyperparathyroidism. It consists of placing the patient on a diet low in phosphorus (350 mg/day), but adequate in calcium and caloric content, and observing the changes in serum calcium, serum phosphorus and urinary phosphorus. Chambers *et al.* (1956) and Goldzieher *et al.* (1957) reported their experiences with it but the test remains to be fully evaluated. As a test with general applicability it does not strike the present author as being very promising; however, it may have a particular use in unmasking a case of hyperparathyroidism in which the serum levels of calcium and phosphorus (especially the latter) may not be notably abnormal.

4. Tubular Reabsorption of Phosphorus

Crawford and his colleagues (Crawford *et al.*, 1950; Talbot *et al.*, 1952) have suggested that the ratio TRP/GFP*, calculated from measurements of the serum inorganic phosphorus and inulin or creatinine clearance, is a useful index of parathyroid activity. This ratio was found to be 0 86 and 0·88 for 2 normal subjects on their usual diet, and it was affirmed that the ratio approached 0 0 and 1·0 under conditions of hyperparathyroidism and hypoparathyroidism respectively. Although the authors supported this claim with no clinical evidence of their own, they did state that "recalculation of extensive clinical data from the literature has shown that the behaviour of this index, TRP/GFP, is consistent with the presumed parathyroid status of the patients described" (Crawford *et al.*, 1950.) However, clinical support came subsequently from the work of several investigators (Schaaf and Kyle, 1954; Chambers *et al.*, 1956; McGeown, 1957; Thomas *et al.*, 1958; Goldzieher *et al.*, 1957; Kyle, Schaaf and Canary, 1958) who have found mean values of the ratio, TRP/GFP, in normal subjects, to be between 0·85 and 0·91, and in patients with hyperparathyroidism, to be significantly lower, 0·58 to 0·85 (Table 7.3). Furthermore, in those patients in whom the ratio was determined post-operatively the values, in general, increased. The chief merit of the test is that it rarely gives a false negative result in cases of hyperparathyroidism†; the main drawback of the test is its lack of specificity, for false positive results may be obtained in conditions other than that of hyperparathyroidism. In 10 patients with hypercalcemia of aetiology other than hyperparathyroidism values for the ratio of 0·56 and 0·58 have been reported (McGeown, 1957; Thomas *et al.*, 1958). This test rests on the assumption that the renal tubule does not secrete phosphorus and that the tubular reabsorption of phosphorus is under parathyroid control. These assumptions must now be viewed with suspicion, following the recent important observation that the distal tubule of the dog kidney is capable of secreting phosphorus (Nicholson and Shepherd, 1959) and that this secretion, and not the reabsorption occurring in the proximal tubule, is influenced by the parathyroids (Nicholson, 1959).

The test is of no use in the diagnosis of hypoparathyroidism, as is indicated by the data in the literature presented in Table 7.3. This was one reason which led Kyle, Schaaf and Canary (1958) to suggest a modification of the test whereby the measurement of the phosphorus clearance only is used as an index of

* TRP/GFP: tubular reabsorbed phosphorus/glomerular filtered phosphorus.

† If a value for the TRP/GFP ratio of 0·82, or less, is taken arbitrarily as indicative of hyperparathyroidism, then of the 41 cases recorded in Table 7.3 only 3 gave false negative results.

Lactation, on the other hand, causes a severe drain on calcium stores. The bones of rats have been found to undergo considerable reduction both in total weight (Fournier and Susbielle, 1953) and in ash content (Ellinger *et al.*, 1952) during lactation. The latter authors demonstrated that this loss occurred regardless of the dietary calcium intake which in their experiments ranged from 0·04 to 0·79 per cent. Thus, the mild hyperparathyroidism which exists in pregnancy becomes more severe during lactation, resulting in greatly increased resorption of bone. This has to be reconciled with the fact that in humans (Knapp and Stearns, 1950) and in rats (Fournier and Susbielle, 1952*a*, *b*) the urinary excretion of calcium decreases during lactation. However, such an observation is consistent with that of Talmage and his associates (Talmage and Kraintz, 1954; Talmage *et al.*, 1955; Talmage, 1956), who have shown, in the rat, that calcium excretion in the urine decreased following administration of parathyroid extract, and increased after parathyroidectomy. Increased activity of the parathyroids does not seem to be the only adaptive response to the stress of lactation, for it has been demonstrated in rats that increased absorption of calcium from the intestine occurred during lactation whether the rats were maintained on a diet low in calcium (Fournier and Susbielle, 1952*b*) or rich in calcium (Fournier and Susbielle, 1952*a*). On the low calcium diet (0·23 per cent Ca) the absorption of calcium increased from 18–48 per cent of the intake during gestation to over 90 per cent during lactation, while on the high calcium diet (0·83 per cent) the absorption of calcium increased from 4–12 per cent during gestation to 50–70 per cent during lactation. McCarthy, Evans and Dragstedt (1954) suggested that the increased volume and free acidity observed by them to occur in dogs, as a result of lactation, may be an important factor in facilitating the absorption of calcium from the gastro-intestinal tract. However, the possible role of Nicolaysen's endogenous factor or parathyroid hormone in this regard must not be overlooked.

B. Diagnosis of Parathyroid Dysfunction

Clinical investigators involved in the diagnosis and treatment of disorders of calcium metabolism are constantly seeking more reliable indices of parathyroid dysfunction. It is often very difficult to make a diagnosis based only on the rather non-specific clinical symptoms, and the serum levels of calcium and inorganic phosphorus. For this reason several tests have been proposed and used with varying degrees of success. It is safe to say that the ideal test has not yet been found. One of the first attempts to devise a diagnostic aid was the test described by Ellsworth and Howard (1934). However, the Ellsworth–Howard test has not proved completely reliable, as has been discussed elsewhere (Greep and Kenny, 1955; Munson, 1955). More recent attempts have included the following: (1) the calcium deprivation test, (2) the calcium infusion test, (3) the phosphorus deprivation test, (4) the determination of the tubular re-absorption of phosphorus and, a modification of the latter, the estimation of the phosphorus clearance, (5) the cortisone test. The details of these tests (except the last one) and of other aids to the diagnosis of parathyroid disease have been set forth by Reifenstein (1958).

1. Calcium Deprivation Test

For the diagnosis of hyperparathyroidism, this test, described by Albright and Reifenstein (1948), is probably the most reliable. It requires placing the patient on a low calcium diet for one week and measuring the 24-hr output of calcium in the urine. The urine calcium of normal persons falls within 3 days to values of 125 mg/day or less. A value of more than 150 mg/day is considered suggestive of hyperparathyroidism. The test has proved reliable even in patients with renal pathology. In 8 patients with proved hyperparathyroidism and renal calculi, determination of the 24-hr urine calcium levels while the patients were on a low calcium diet (154 mg/day) gave a mean value of 256 mg with a range of 141–439 mg (McGeown, 1957). In 3 patients with elevated serum calcium levels, but with no evidence of parathyroid pathology on surgical exploration, a mean value of 95 mg was found with a range of 59–133 mg.

2. Calcium Infusion Test

First proposed by Howard, Hopkins and Connor (1953) the calcium infusion test derives from the work of Baylor *et al.* (1950), who had noted that the intravenous administration of calcium as calcium gluconate into normal subjects resulted in a decrease in urinary excretion of phosphorus. The former group made a detailed study of the changes in serum and urine phosphorus following infusion of calcium in normal persons, and in cases of hyperparathyroidism, and of surgical hypoparathyroidism. Infusion of calcium, 15 mg/kg over a 4-hr period, to 8 normal subjects resulted in a fall in urinary secretion of phosphorus within the 24-hr period following the start of the infusion. The fall in urinary phosphorus excretion occurred in spite of a concomitant rise in serum inorganic phosphorus. A similar test carried out in 5 hyperparathyroid patients gave different results. Two patients showed a slight fall in phosphorus output; 2 patients exhibited no response; while 1 patient showed a rise in phosphorus output. Three cases of surgical hypoparathyroidism all responded to the test by a marked rise

plastic disorders which may occur in human parathyroid tissue. Hyperparathyroidism may be either primary or secondary in nature. The former includes adenomas, carcinomas and primary hyperplasia and hypertrophy, while the latter consists of hyperplasia resulting from chronic renal insufficiency and consequent retention of phosphate. By far the most common form of primary hyperparathyroidism is the adenoma which occurs in about 89 per cent of the cases (Castleman, 1952; Black and Zimmer, 1956). The condition of primary hyperparathyroidism is always accompanied by an elevated level of ionic calcium in the serum, which almost invariably reflects itself in an increased total calcium in the serum. However, as pointed out by Lloyd and Rose (1958), this is not necessarily so, as these authors were able to demonstrate that in 2 out of 17 cases of primary hyperparathyroidism the total calcium was within normal limits (10·1 and 10·4 mg/100 ml). As a result of the elevated level of ionic calcium there is a suppression of nerve and muscle activity. The majority of cases of primary hyperparathyroidism involve renal disease, to the extent of 74 per cent in Hellstrom's (1954) series of 50 cases, and of 82 per cent in a series of 207 cases at the Mayo Clinic (Black and Zimmer, 1956). This is, of course, partly a reflection of the fact that patients with renal calculi are, or should be, routinely screened for possible hyperparathyroidism. It is important to note that at least 5 per cent of all patients exhibiting renal calculi have a co-existing hyperparathyroidism (Castleman, 1952), for in these cases early diagnosis and treatment of the parathyroid disease can prevent further renal damage. Bone disease, as would be expected, is frequently associated with the endocrine disorder. The skeletal changes described as osteitis fibrosa generalisata were found by Hellstrom (1954) to be present in 46 per cent in his cases. Thus, clinical types of primary hyperparathyroidism may be classified into those with bone disease and those without bone disease, but perhaps this is only a quantitative separation rather than a qualitative one. It was this fact, among other considerations, which led Kenny and Castleman to undertake a study of the hormonal content of human parathyroid glands, both normal and neoplastic. It was thought that this clinical classification might reflect itself in the ratio of the hormonal activities, calcium-mobilizing/phosphaturic, of extracts prepared from tumors from the two clinical types. The groundwork has been laid, in that extracts of normal glands have been prepared and shown to be active in the parathyroidectomized rat (Kenny and Castleman, unpublished). In addition, active extracts of adenomas, and of primary and secondary hyperplasias have been prepared. However, it has not been possible to obtain consistently active extracts of either normal or neoplastic parathyroids. It is conceivable that crude extracts of human parathyroids are more unstable than those of bovine origin. An extract of human parathyroid adenomas has been shown to respond to activation by cysteine so that spontaneous inactivation had probably occurred.

An interesting, though unexplained, symptom which not infrequently occurs in cases of hyperparathyroidism (particularly among men) is that of duodenal ulcer. Hellstrom (1954) found this particular symptom to the extent of 14 per cent in his series.

D. Hypoparathyroidism

The clinical condition of hypoparathyroidism may arise either accidentally, by the removal of normal parathyroids during thyroid surgery, or spontaneously in the disorder known as idiopathic hypoparathyroidism. In the latter condition, first described by Albright and Ellsworth (1929) and later more fully by Drake *et al.* (1939), the parathyroids though present must be non-functioning. The clinical aspects of the disease have been amply characterized by Albright and Reifenstein (1948). However, some confusion exists as to whether or not the syndrome reported by Albright *et al.* (1942), and called by them pseudo-hypoparathyroidism, is an entity separate from idiopathic hypoparathyroidism. The differential diagnosis is based largely on the lack of response the pseudo-hypoparathyroid patient exhibits to the Ellsworth–Howard test, a test which, as has already been pointed out, is not completely reliable (Greep and Kenny, 1955). Thus, Albright *et al.* (1942) suggested that the patho-physiological basis for the difference between the syndromes lies in the phenomenon that, in pseudo-hypoparathyroidism, the patient is resistant to the activity of the hormone endogenously secreted. Robinson, Carmichael and Cumings (1954) questioned the separability of the two syndromes and, in fact, classified the condition of three patients who had failed to respond to the Ellsworth–Howard test as idiopathic, not pseudo-, hypoparathyroidism.

There are continuing reports in the literature of attempts to treat hypoparathyroidism by homotransplantation of parathyroid tissue. Early attempts utilized fragments of parathyroid tissue which had been maintained in tissue culture medium containing recipient serum (Stone, Owings and Gey, 1934). The partial success (20 per cent) of this technique has been confirmed by Gaillard (1954) and by Escamilla *et al.* (1957). A more promising technique is the total homotransplantation of the thyroid and parathyroid glands using vascular anastomoses (Sterling and Goldsmith, 1954). This technique, when compared with three other methods of transplantation, was the most

TABLE 7.3

Tubular Reabsorption of Phosphorus as an Aid in the Diagnosis of Parathyroid Dysfunction
(Survey of the literature)

Diagnosis	No. of patients	Ratio TRP/GFP		Authors
		Range	Mean	
Normal	2	0·86–0·88	0·87	Crawford *et al.* (1950)
	10	*	0·91	Schaaf and Kyle (1954)
	13	0·78–0·90	0·85	Chambers *et al.* (1956)
	7	0·84–0·91	*	Thomas *et al.* (1958)
Hyperparathyroidism	3	0·49–0 65	0·58	Schaaf and Kyle (1954)
	10	0·41–0·82	0·65	Chambers *et al.* (1956)
	3	0·76–0·99	0 85	Goldzieher *et al.* (1957)
	8	0·55–0·86	0·66	Thomas *et al.* (1958)
	6	0·49–0·75	0·64	Kyle *et al.* (1958)
	11‡	0·63–0·88	0·75	McGeown (1957
Post-operative hyperparathyroidism	1	0·98	0·98	Schaaf and Kyle (1954)
	9	0·98–1·00	*	Chambers *et al.* (1956)
	2	0·94	0·94	Goldzieher *et al.* (1957)
	1	1·00	1·00	Kyle *et al.* (1958)
	10‡	0·53–0·99	0 86	McGeown (1957)†
Hypoparathyroidism	22	0·74–0·99	0·89	Chambers *et al.* (1956)
	7	0·90–0·98	0 93	Kyle *et al.* (1958)
Hypercalcemias without hyperparathyroidism	7	0·37–0·81	0·58	Thomas *et al.* (1958)
	3	0·27–0·80	0·56	McGeown (1957)

TRP/GFP=tubular reabsorbed phosphorus/glomerular filtered phosphorus.

* Data not given by the authors.

† Data originally presented as C_p/C_{cr} (phosphorus clearance/creatine clearance) were converted to to the TRP/GFP ratio using the relationship, TRP/GFP$=1-C_p/C_{cr}$.

‡ *All these patients had renal calculi.*

parathyroid function. In 25 normal subjects a mean value of 10·8 (standard deviation ±2·7, range 6·3–15·5) ml/min was obtained for phosphorus clearance. In 10 patients with hypoparathyroidism the phosphorus clearance was diminished to a mean value of 5·0 (range 1·7–7·3) ml/min, whereas the clearance was elevated in 6 patients with hyperparathyroidism (mean 27, range 13–40 ml/min). Of significance is the fact that vitamin-D therapy in hypoparathyroid patients had little effect on the value of phosphorus clearance.

4. Cortisone Test

The fact that cortisone administration lowers the level of serum calcium in several hypercalcemic states (Anderson *et al.*, 1954; Connor *et al.*, 1956), with the exception of hyperparathyroidism, led Dent (1956) to propose a test based on this phenomenon for the differential diagnosis of hyperparathyroidism. The procedure consists in administering cortisone, 150 mg/day, for 10 days, during which time serum calcium determinations are made on days 5, 8 and 10. The test possesses two main disadvantages. First, there is the possibility of precipitating an *Addisonian crisis after* withdrawal of the cortisone treatment (Dent, 1956), and secondly, the test can give false positive results (Thomas *et al.*, 1958).

C. Hyperparathyroidism

In an excellent monograph, Castleman (1952) has reviewed the classification and pathology of the neo-

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successful (88 per cent) in the dog, when judged by morphological evidence of viable parathyroid transplant (Jordan, Foster and Gyorkey, 1958). In order to minimize variables, autotransplants were used in this study. Two hypoparathyroid patients were mentioned by these authors as having benefited from receiving homotransplantation of the thyroid and parathyroid glands by this technique. The direct homotransplantation of foetal or newborn parathyroid tissue into the rectus abdominis muscle has proved beneficial in at least two cases (Rigdon and Mead, 1956; Akers, Binkley and Miller, 1958).

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Vitamin-D Deficiency and Bone and Tooth Structure

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Contents

I. INTRODUCTION

Vitamin-D deficiency, due to a diet containing an insufficient amount of vitamin D, or resulting from inadequate absorption of the vitamin, leads to disturbances in the normal calcification process of the hard tissues of the body and, consequently, to characteristic pathological changes. In the young growing subject, lack of vitamin D causes a disease known as rickets, whereas the organism affected by vitamin-D deficiency, after length growth and dentition have been completed, is subject to a disorder called osteomalacia. Rickets has, in former times, constituted a serious clinical problem because of its high incidence. Ritchi (1871), for instance, reported that grave rachitic changes were seen in one-third of all children under 5 years of age admitted to his service in Manchester. Schmorl (1909), in his histological study of the growth zone of the long bones in infants dying 2–48 months after birth, stated that he observed gross or microscopic changes indicative of active rickets in 73 per cent of 386 children. By including the cases with changes characteristic of healed rachitic lesions, this incidence was increased to 89 per cent. In civilized countries, changing social and hygienic conditions, together with adequate prophylaxis, have gradually been effective in lowering this rate considerably. As recently as 1952, however, painstaking microscopic examination of autopsy material by Follis *et al.* yielded a 68 per cent incidence of rachitic manifestations in children dying within 11 months after birth. Nevertheless, from the clinical point of view, rickets is, at the present time, considered extremely rare in civilized countries. Under less favourable conditions, however, with poorer nutrition or neglect of prophylaxis, there would be a great risk of the disease attaining considerable clinical significance. This is emphasized by a study conducted during the second World War by the British Paediatric Association for the British Ministry of Health (1944). Five thousand school children were examined for clinical and roentgenologic signs of rickets. It was found that "the incidence of rickets diagnosed radiologically in children between 3 and 18 months of age is 2·5 per cent before 6 months, 4 per cent in the first year and negligible after that period." This would seem to be a low incidence but for the fact that more effective prophylaxis should have reduced the incidence to approximately zero.

Despite adequate nutrition and favourable hygienic conditions, rachitic and osteomalacic lesions are still encountered, if only sporadically. In many cases they are caused by incomplete utilization of vitamin D as, for instance, in steatorrhea, where the absorption of fat and fat-soluble vitamins is impaired. In a small group of cases, however, rachitic changes may become manifest despite normal intake and absorption of vitamin D. In this category belong a heterogeneous group of symptoms classed together under the name of vitamin-D resistant rickets or osteomalacia.

Even if rickets or osteomalacia under normal conditions is rare, it is obvious that these diseases could be expected to become prevalent if the prophylactic efforts were to be relaxed. In addition, there is the possibility of rickets as a sequela of steatorrhea and, furthermore, the cases of vitamin-D resistant rickets should be taken into account. It would, seem, therefore, that the clinical management of rickets still remains an actual problem. Further considerations are the divergence of opinions prevailing as to certain aspects of the mode of action of vitamin D, as well as the need for more complete explanation of the changes in bone and dental tissues as caused by vitamin-D deficiency.

II. VITAMIN-D DEFICIENCY STATES IN THE GROWING INDIVIDUAL

A. History

Rickets is a disease of childhood, predominating in the ages between 6 and 24 months, and is reflected most prominently by skeletal and dental deformations. These changes are characteristic of an impaired calcification of bone structures which normally calcify, resulting in fairly typical pathological changes in the hard tissues. The defective calcification of the organic matrix leads to decreased resistance to mechanical strains, which ultimately results in deformities of the hard tissues. Two explanations prevail as to the origin of the name "rickets." According to one theory, the name has its origin in the English Dorset dialect where "to rucket" means "to breathe with difficulty." Another explanation is that the name should be derived from the Greek word for spine, which is rachis (ῥαχις). The term rachitis, which is the denotation prevailing on the European continent, is also considered to be a derivative of this word (Hess, 1930; Nicolaysen and Eeg-Larsen, 1953).

The first adequate description of rickets is attributed to Soranus of Ephesus, in the first century after Christ. He stated that a deformation of the spine and the bones may occur at the time when a child learns to

histological study of rickets appearing inadvertently in experimental rats, it was established that the disease could attack these animals. McCollum *et al.* (1920–21) and Sherman and Pappenheimer (1920–21, 1921), basing their studies on this fact, and on their personal observations of rickets occurring in animals which differed from normal specimens only with respect to diet, succeeded in producing the disease experimentally in rats by modifying the diet. Like Mellanby, they also adopted the most obvious explanation of vitamin A as the antirachitic principle. This theory had to be abandoned after only a short time, however, once it had been established that, although xerophthalmia in rachitic animals was affected by butter fat, this had no appreciable effect on rachitic lesions in the skeleton (Shipley *et al.*, 1920–21*a*; McCollum *et al.*, 1921*a*). A study made on rachitic rats, in order to determine the effect of various fats with respect to xerophthalmia and growth, and their efficacy in healing rickets, finally established conclusively that vitamin A was not the protective agent in rickets; regression of the disease was achieved by supplying the animals with another vitamin (vitamin D) which was considered to regulate bone metabolism (McCollum *et al.*, 1922).

Recognition of rickets as a vitamin-D deficiency gave rise to a new trend in research, directed at isolating this vitamin. One of the starting points for these investigations was the prevalence of rickets in months with only a few hours of sunshine, a long-established fact. A study of this phenomenon was made by Huldchinsky, who, in 1920, was able to demonstrate that rickets in children could be healed by exposing them to the ultra-violet rays of a quartz lamp. Hess and Weinstock (1924) and Steenbock and Black (1924) subsequently showed that it sufficed to irradiate the food eaten by rachitic rats. These observations, together with the long familiar fact that cod-liver oil was an excellent remedy for rickets (Schabad, 1910; Schloss, 1916*a*, *b*), led to recognition of at least two different variations of vitamin D, the most important of which are vitamin D_2 and vitamin D_3. The former, also called calciferol, is produced by ultra-violet irradiation of ergosterol; it was first isolated in purified form by Askew *et al.* (1931) and by Windaus 1931*a*, *b*). Vitamin D_3 is found in fish-liver oil; it is produced artificially by ultra-violet irradiation of 7-dehydrocholesterol. In the animal organism the vitamin develops on ultra-violet irradiation of the animal. For more detailed chemical information, the reader is referred to Deuel (1951).

B. Effect of Vitamin-D Deficiency in Various Species of Animals

Present knowledge of the rachitic changes in the structure of the hard tissues is in part derived from studies on human material, partly also obtained through animal studies of both spontaneous and experimental rickets. The animals subject to rachitic changes include dogs, rats, hens, pigs, lambs, kids, and calves (Harris, 1956). Dogs, hens and rats have been predominantly used for experimental studies of rickets.

In children, rickets is liable to occur if the diet is deficient in vitamin D, even though the supply of calcium and phosphorus may be relatively normal (Snapper and Nathan, 1957). The same conditions prevail in dogs, as demonstrated by Mellanby's experiments (1921), in which he could not prevent rickets in puppies by adding calcium phosphate to an otherwise rachitogenic diet. Attempts to produce experimental rickets in rats, on the other hand, have shown that vitamin-D deficiency in itself does not suffice to provoke the disease in these animals. Sherman and Pappenheimer (1920–21), Shipley *et al.* (1920–21*a*), McCollum *et al.* (1921*a*) all produced experimental rickets in rats by feeding them a diet poor in vitamin D, and containing normal to ample amounts of calcium, but a slight quantity of phosphorus. By adding phosphorus, in the form of phosphate, in sufficient amounts to bring the initially high Ca/P quotient in the diet down to normal, they managed to prevent rickets in these animals. McCollum *et al.* (1921*b*) were also able to produce experimental rickets in rats by feeding them a diet with an abnormally low Ca/P quotient. Shohl and Wohlbach (1936) also demonstrated that either an abnormally high or low Ca/P quotient could produce rickets. However, if the absolute quantity of calcium and phosphorus in the food was extremely small, rachitic changes could occur despite a normal Ca/P quotient in the diet. From the investigations discussed in the foregoing, it is evident, therefore, that, in rats, vitamin-D deficiency requires the additional factor of a diet with an abnormal Ca/P quotient, or of an insufficient amount of calcium and/or phosphorus in the diet, in order to produce rickets.

Metabolic studies on human material, dogs and rats have shown that in the two first named, the absorption of calcium, in vitamin-D deficiency states, differs from that in rats. Schabad (1910) and Schloss (1916) at an early stage demonstrated a loss of calcium salts in the faeces in human rickets, resulting in a negative or significantly lowered positive calcium balance. If cod liver oil was supplemented, the amount of calcium salts in the faeces was reduced and a positive balance regained. Similar observations have been made in dogs (Mellanby, 1949). In rats, on the other hand, absorption of calcium from the intestine is considerable, despite lack of vitamin D in the diet (Nicolaysen and Eeg-Larsen, 1953). In studies on

walk or to sit, a condition possibly caused by softening of the bones. Several centuries passed, before, in the 16th and 17th centuries, the disease was again adequately described, this time by a small number of medical writers on the European continent. In the middle of the 17th century three important works on rickets appeared in England. In 1645, Whistler published a thesis on the disease, followed in 1649 by a paper on the same subject by Bootius. Their works appear almost compendious, however, in comparison with a subsequent paper by Glisson (1650), which is the first to give a more detailed account of the disease. Through this pioneer work, Europe's medical experts were alerted to the disease which, either because its most exhaustive description had come from England, or because of its high incidence in that country at the time, received the name "English disease."

Several important contributions on the pathological anatomy in rickets were added in the 19th century. Rufz (1834), describing gross pathological signs observed in rachitic children, pointed out that, in addition to the bones being softened, the growth zone in the long bones had a widened and irregular appearance. In 1837, Guérin, lecturing before the French Academy, was the first to observe that three different stages of development could be encountered in rickets, with varying clinical and pathological features. According to him, the first stage is reflected only by a softening of the bone tissues followed by thickening of the epiphysis in some areas in the latter part of this stage. In the second stage, deformities become apparent, whereas renewed hardening of the tissues is found in the third and last stage. A subsequent paper by Elsässer (1843) indicated craniotabes as one of the earliest signs of rickets. Contributions by Köllicker (1847), Meyer (1849), Virchow (1853) and Müller (1858) shed further light on the gross pathological anatomy, and added to medical knowledge, by describing some of the histological changes. A really outstanding work was published by Pommer (1885). From his scrupulous histological studies of bone tissue, he concluded that formation and resorption of bone tissue continued throughout life. The essential characteristic of rickets was, in his opinion, that normally-formed new bone tissue failed to become calcified, resulting in excessive accumulations of osteoid. Consequently, softening of the bone tissue was not caused by increased absorption of bone tissue. Schmorl (1909) further emphasized the significance of osteoid in rickets, and gave detailed descriptions of the histological changes encountered in both active and healing rickets.

Concurrently with these studies on the pathological anatomy, other scientists were investigating the chemical features of bone tissue. Marschand (1842) and Friedleben (1860) concluded from such investigations that rachitic bones, in comparison with healthy bones, contained smaller amounts of bone salts, consisting chiefly of calcium and phosphorus, whereas the water content was larger than normal.

Pathological and chemical studies consequently established, at an early stage, that rickets is associated with a calcium deficiency in the bone tissues. Since a disturbance in the supply of calcium to the organism could be considered the most obvious explanation, attempts were made to produce rickets in animals by experimental methods. As early as 1842 and 1861, Chossat and Edwards, respectively, had succeeded in producing a fragile, easily-fractured skeleton in pigeons by feeding them a diet deficient in calcium. Voit (1880) and Baginsky (1882), feeding puppies on meat and lard, which are poor in calcium, produced changes suggestive of rickets, including widening of the growth zones. After Pommer in his subsequent papers had stressed the role of osteoid in rickets, however, it was questioned whether these authors in fact had produced rickets in their experimental animals. Miwa and Stoeltzner (1898), Aron and Sebauer (1908), and Dibbelt (1909) therefore carried out new experiments and managed to produce changes suggestive of rickets, in some cases with osteoid surrounding the trabeculae. Miwa and Stoeltzner (1898), however, considered these changes indicative of an osteoporosis resembling rickets rather than of genuine rickets. The same conclusion was reached by Götting (1909) in his microscopic study of the bone tissue from the dogs used in Aron and Sebauer's experiments.

Eijkmann's (1897) successful attempt to produce beriberi in pigeons, together with Holst's and Froehlich's experiment (1912) in which they managed to produce scurvy in guinea-pigs, suggested the idea that unknown specific nutritional factors, so-called vitamins, influenced certain metabolic processes in the organism. The problem of rickets was approached along the same lines, and the possibility of rickets being a vitamin-deficiency disease was advanced. This led to more consistent attempts to produce rickets experimentally by modifying the diet. In 1921, Mellanby published a long and detailed account of his successful efforts to produce rickets in dogs by feeding them a diet deficient in certain fats. He also succeeded in preventing rickets by supplementing the animals' rachitogenic diet with these fats, which included cod-liver oil and butter fat, but no vegetable fats. Since these antirachitic principles were found in various fats, Mellanby was led to believe that the protective element was provided by vitamin A, which was known at that time. Concurrently with Mellanby, American scientists were attempting to produce rickets in rats. Through Erdheim's (1914) careful



PHOTOMICROGRAPHS OF 5-μ THICK MICROTOME SECTIONS FROM A 3-MONTHS-OLD DOG

(*a*)—Decalcified growth zone. Epiphyseal cartilege with proliferation zone and hypertrophic zone; below this, calcification zone and metaphysis with invading capillaries (×96). (*b*)—Similar area to (*a*) Specimen taken from dog with severe rickets. Note the irregular vascular invasion Htx-eos (×96).

B

MICRORADIOGRAM 10–50 Å OF 5-μ THICK UNDECALCIFIED SECTION OF COSTOCHONDRAL JUNCTION

(*a*)—Normal growth zone (×96) (*b*)—Part of hypertrophic cartilage zone and adjoining bone tissue from rachitic animal. Normal mineralization of the provisional calcification zone is absent, but higher up in the epiphyseal plate a small mineralized area is evident (×96)

PLATE V

chickens (Wanscher, 1939; McChesney and Giacomino, 1945), calcium absorption in these animals was found to be similar to that in children and dogs.

From the foregoing it appears that calcium metabolism in rats differs from that in man, and from that in other animals commonly used in rickets research. In chickens, calcium absorption appears to be similar to that in humans, but the structure and metabolism of bone are different. The greatest similarity with respect to calcium metabolism in rickets is exhibited by children and dogs; on comparison of the animals commonly employed in experimental research in this field, the bone tissue in dogs is seen to bear the greatest resemblance to that of man. The structural changes of the hard tissues observed in rachitic conditions are essentially alike for the different species. In evaluating the structural changes in human ricket versus those seen in rachitic experimental animals, due consideration should, however, be given to the fact that calcium metabolism and bone structure vary in the different species.

C. Gross Pathology

The gross alterations of bone in rickets have to some extent been described as early as 1650, by Glisson. In the 19th century, valuable contributions were made by several authors, among others Rufz (1834), Elsässer (1843), Köllicker (1847), Virchow (1853), Guérin (1862) and Pommer (1885). Their combined efforts succeeded in elucidating the gross pathological changes caused by rickets. A common feature of all rachitic conditions is a softening of the bone due to a diminished deposition of calcium salts. The earliest change in rickets is seen in the skull; this is called craniotabes. It was first described by Elsässer (1843). Craniotabes denotes a condition in which the bone tissue surrounding the lambdoid suture, on palpation, is found to be thin and poorly calcified. On the other hand, the bone tissues may be abnormally thickened in other areas, in particular at the ossification centres of the frontal and parietal bones. These hypertrophic areas give the skull an appearance that is characteristic of rickets, and is known as caput quadratum. Fontanelles, as well as sutures, are often abnormally wide.

The long cylindrical bones show varying degrees of deformation, depending upon the severity and the duration of the disease. A common feature is that the normal curvatures of the cylindrical bones tend to be more pronounced. This may be accompanied by other deformities. The strain put upon the bones by the body weight when the child starts to walk is apt to cause, in the legs, deformities of such severity that knock-knee or bow-legs result. In such cases, however, the deformities are not restricted to the bones; even the joint surfaces between femur and tibia have changed to such extent that the medial and lateral parts of the cartilage are no longer equally thick. Pathological fractures are seen in some cases. They may be so serious that the bone is completely severed, although so-called "green-stick fractures" are also common. In the latter type of fracture, the fracture line extends from the surface of the cortex into the medullary areas. Callus formation in these fractures is more abundant than normal.

A characteristic rachitic change, which early attracted general attention, is an increase in width of the epiphysis and the metaphysis. Longitudinal section of the bone shows this widening to be due to thickening of the epiphyseal cartilage which, instead of its normal straight outline at the metaphysis, has an irregular appearance in this area, a phenomenon first described by Rufz (1834). Under the thickened epiphyseal cartilage lies a soft, greyish mass of tissue, known as the rachitic metaphysis, which will be discussed in more detail in a later section. The periosteum is sometimes thickened, and covers a soft, newly-formed tissue that has not calcified. The tissue in the marrow cavity looks redder than normal, which led Kassowitz (1881, 1884), among others, to believe that rickets was an inflammatory disease due to the effect of a toxin. As a result of impaired growth at the epiphyseal plates the long bones may become shorter than normal (*i.a.* Wilton, 1937). The extent of this shortening depends on the intensity of the disease and upon its duration.

The most typical change in the thorax is the appearance of the so-called rachitic rosary, consisting of a succession of prominences along the border between costal cartilage and bone. These prominences may be so minute that they are easily missed on clinical examination; in particular, since they predominantly occur on the inside, facing the thoracic cavity. In other cases the changes may be so pronounced that they are strongly conspicuous and easily palpable.

Another deformity at times observed in rickets is a groove (Harrison's groove) along the lower border of the thorax, caused by the malleable ribs giving way under the muscle-pull exerted by the diaphragm at its line of attachment to the thoracic wall. In extreme cases, the ribs may be so soft that they give way under the negative force which develops in the thorax on inspiration. This type of deformation may constitute a vital risk for the patient and, at times, even cause death (Park and Howland, 1921). In such severe cases the sternum projects farther than normal, while the lateral parts of the thorax approach each other, thus decreasing the chest volume. The appearance of the sternum in such cases has originated the name "chicken breast."

In serious cases of rickets, kyphotic or scoliotic deformities of the spine may appear, accompanied by



(*a*) MICRORADIOGRAM 1–3 Å OF A 100-μ THICK GROUND SECTION FROM 3-MONTHS-OLD RACHITIC DOG

The considerably widened epiphyseal plate is seen as a black area. Below this, the irregular bone trabeculae in the metaphysis (×20).



(*b*) MICRORADIOGRAM 10–50 Å OF UNDECALCIFIED 5-μ THICK MICROTOME SECTION OF METAPHYSEAL BONE TISSUE

Showing mineralized areas and osteoid covered by osteoblasts The osteoid tissue appears rather dark in the picture (×100)

PLATE VII

A



Microradiogram 10–50 Å of 5-μ Thick Decalcified Microtome Section comprising the Lower Part of the Epiphyseal Plate and Adjoining Parts of the Metaphysis

(*a*)—Normal growth zone (×100). (*b*)—Epiphyseal plate from rachitic animal The irregularity of the hypertrophic cartilage zone and the absence of a zone of "clear cartilage cells" are conspicuous; likewise, the coarseness of the bone trabeculae in the metaphysis (×100).

B



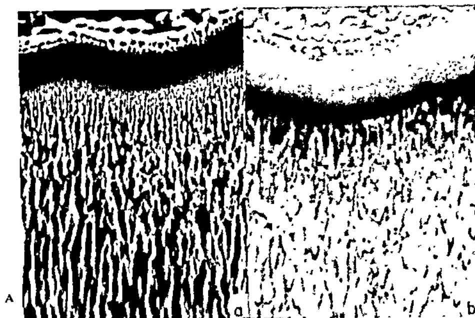
(*a*) Microradiogram 10–50 Å of Undecalcified 5-μ Thick Microtome Section containing so-called Pseudo-osteoid

The calcified regions in the matrix and in and around the cartilage cells are visible as lighter areas in the picture (×100).

(*b*) Microphotograph of the Histologic Section Microradiographed in (*a*) (×100).

Plate VI

A



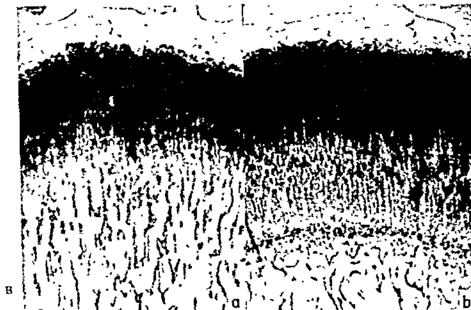
(*a*) MICRORADIOGRAM 1–3 Å OF 100-μ THICK GROUND SECTION OF GROWTH ZONE FROM NORMAL 3-MONTHS-OLD DOG, SHOWING THE DISTRIBUTION OF MINERAL SALTS

Except for the mineralized matrix in the zone of the provisional calcification, the epiphyseal cartilage has no demonstrable absorption of the roentgen rays and is consequently seen as a black area in the picture. Below this area the provisional calcification zone is seen, and below that the bone tissue in the metaphysis (×12½)

(*b*) AUTORADIOGRAM. RADIOCALCIUM GIVEN AFTER THREE DAYS PRIOR TO DEATH. SAME SECTION AS IN (*a*)

No uptake in the epiphyseal cartilage, except in the provisional calcification zone. The uptake is most pronounced in the metaphysis adjoining the calcification zone of the cartilage (×12½)

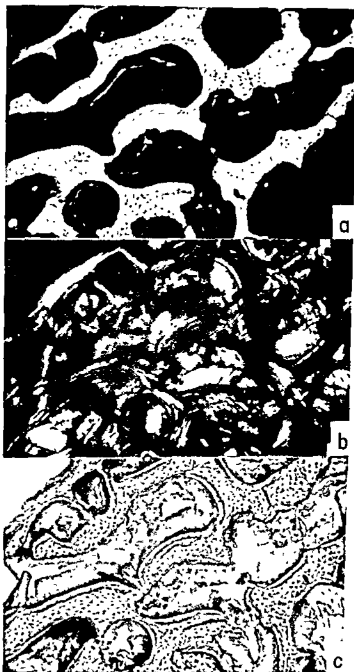
B



AUTORADIOGRAM (RADIOSULPHATE GIVEN 3 DAYS PRIOR TO DEATH) OF 5-μ THICK MICROTOME SECTION OF DECALCIFIED BONE TISSUE COMPRISING THE EPIPHYSEAL PLATE AND ADJOINING PARTS OF THE METAPHYSIS

(*a*)—Normal pattern of uptake. Note the distinct labelling in the epiphyseal plate decreasing towards the metaphysis. An appreciable uptake is also apparent in the peripheral parts of the bone trabeculae (×25).
(*b*)—Similar specimen to (*a*) but from rachitic dog. Note the greatly widened epiphyseal plate and the irregular distribution of the radioactivity, the greater part of which is seen in the upper region of the epiphyseal plate. Bordering upon the metaphysis a narrow zone of marked uptake is visible (×25).

PLATE IX



(*a*) MICRORADIOGRAM 1–3 Å OF 50-μ THICK GROUND SECTION OF THE TIBIAL DIAPHYSIS FROM A RACHITIC DOG

Note the narrow mineralized line in the resorption cavities, separated from the highly mineralized bone tissue by a non-absorbing zone.

(*b*) SAME SECTION AFTER DECALCIFICATION, PHOTOGRAPHED IN POLARIZED LIGHT

The wide osteoid zone shows a fibre pattern which is different from that of the adjacent bone trabeculae.

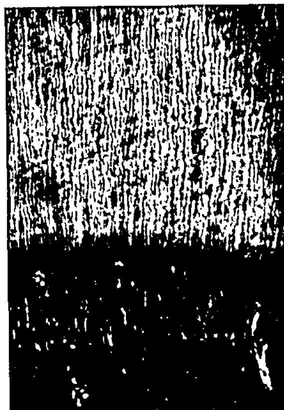
(*c*) SAME SECTION AS IN (*b*), PHOTOGRAPHED IN TRANSMITTED LIGHT

PLATE VIII



(*a*) AUTORADIOGRAM OF ^{35}S LABELLED TISSUE SHOWING PARTS OF THE ROOT OF A TOOTH FROM A RACHITIC DOG

The irregular labelling of the predentine zone is clearly visible, as is the uptake in the interglobular dentine. Decalcified section × 36).

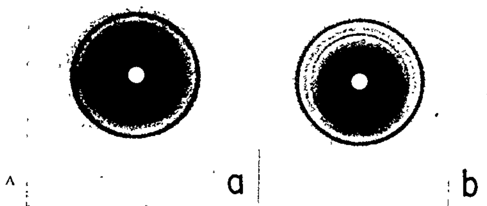


(*b*) MICRORADIOGRAM 10–50 Å OF DENTINE, ODONTOBLASTS AND PULP TISSUE FROM A NORMAL DOG

Decalcified 5-μ thick section. The predentine shows somewhat *less roentgen adsorption than the "mineralized" dentine,* implying a slightly lower dry weight per unit area for the predentine (× 192).

PLATE XI

A



MICRO X-RAY DIFFRACTION DIAGRAMS OBTAINED FROM THE OSTEOID TISSUE SHOWING DEFINITE FIBRING

(a)—shows tissue before heating; (b)—after heating to 600°C.

B

a

b

(a) MICRORADIOGRAM 10–50 Å OF UNDECALCIFIED 5-μ THICK SECTION OF DENTAL ROOT FROM A NORMAL DOG

In the border area between predentine and mineralized dentine mineralization is visible as fine white grains (×288).

(b) SIMILAR TO *(a)* BUT FROM RACHITIC ANIMAL

The defects in mineralization are seen as dark areas of so-called interglobular dentine, i.e. unmineralized areas (×228).

PLATE X

deformation of the thoracic wall and diminished volume of the thorax.

The pelvis may also be subject to severe pathological changes, resulting in the typical rachitic pelvis (Breus and Kolisko, 1904). In such cases the promontory of the sacrum projects, while the acetabulum is displaced inwards towards the pelvic cavity, with the result that the opening to the true pelvis is narrowed. In a pregnancy, this deformity may make parturition difficult.

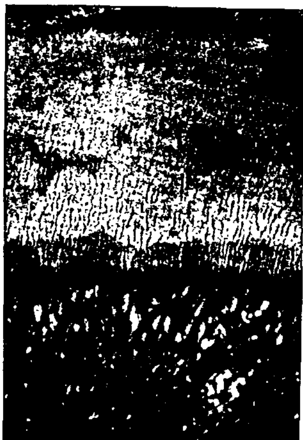
D. Histological Changes

1. The Normal Growth Zone

The study of rachitic changes, demonstrable by microscopic examination, has chiefly been concerned with the growth zones of the epiphyses and ribs. It would, therefore, not be out of the way to precede a discussion of these pathological changes with a description of the normal endochondral ossification processes in mammals. The first to publish an adequate histological account of the process was Müller (1858). Among later, more significant studies, mention should be made of those performed by Dodds (1930, 1932), Dodds and Cameron (1934), and McLean and Bloom (1940).

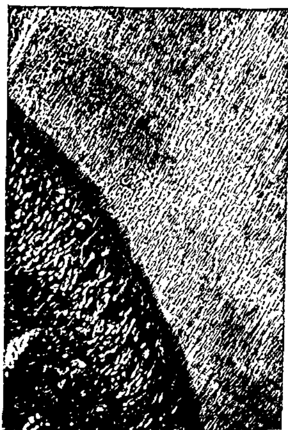
The epiphyseal cartilage can be divided into several zones, *i.e.* the zone of resting cartilage, the zone of cell proliferation, the zone of hypertrophic cells, and the zone of provisional calcification, followed closely by the zone of bone formation, which contains both primary and secondary cancellous bone. Adjoining the epiphysis, cartilage of hyaline appearance is seen. The cells, which in this area are small, vacuolated and rounded, are irregularly and loosely distributed in the basophilic matrix. In this so-called resting cartilage, cell division occurs in all directions. Towards the diaphysis the cells are arranged in parallel columns. The area containing these cell columns is composed of the proliferation zone, the hypertrophic zone, and the provisional calcification zone. From the resting cartilage, newly-formed cells are shifted down to the proliferation zone. They produce the mother cells from which the cell columns are developed. Numerous cell divisions take place in the proliferation zone, and it is here that the increase in length of the bone essentially occurs. On division of the cells in this area, the newly formed cells remain close to each other. The cells are, furthermore, discoid, and oriented in such manner that their long axes lie perpendicular to the long axes of the shafts. Between the individual cells in the cell columns lie thin septa of matrix, with wider areas of cartilage matrix separating the rows of cell columns. The lower part of the zone of cell proliferation constitutes a smooth transition to the next zone containing the hypertrophic cells. In this area, the cytoplasm of the cells is becoming vacuolated, and their nuclei disintegrate. The stainability of both cytoplasm and nuclei is reduced. The hypertrophic process produces a cell of nearly uniform diameter in all directions, instead of the flat, discoid cell seen in the zone adjacent to it (Plate V*A* (*a*)). Throughout the growth of the cells their arrangement in columns is maintained, which, together with the enlargement of the cells, results in the lengthening of these cell columns. In addition the increase in length is achieved, consequently, by enlargement of the cells in the hypertrophic zone. As the cells expand, the cartilage matrix between the cells within the cell columns, and the matrix separating the columns, diminish in size, although the latter continues to be wider than the matrix between the individual cells. Finally, calcification of the cartilage matrix takes place in the zone of so-called provisional calcification, which contains the largest cells. Vacuolation and loss of stainability have made further progress in these cells. Calcification in this zone occurs, according to Dodds (1930) and Dodds and Cameron (1934), exclusively along the longitudinal matrix trabeculae, whereas the thin transverse walls between the cells within the columns remain uncalcified. McLean and Bloom (1940) paid particular attention to the process of calcification in endochondral bone growth by studying sections which, without previous decalcification, were stained with silver nitrate and counterstained with hematoxylin-eosin. They concluded that bone salt is deposited mainly in the longitudinal matrix trabeculae, whereas the transverse, and usually very thin septa between the cells, either do not share in the calcification process at all, or become only partially calcified. They admitted, however, that exceptions do occur, and that it is not uncommon to find adequately calcified cross partitions. Both in this paper and in Freeman and McLean's publication (1941) it was furthermore stated that calcification may, even under normal conditions, be restricted so that it fails to achieve its normal distribution, stretching three or four cells ahead of the capillaries that invade the cells at the lower border of the cartilage. In some cases calcification may be only one cell in advance, or even fail altogether to progress beyond the line formed by the tips of the capillaries.

The cells at the extreme lower end of the cartilage die and are invaded by capillaries from the diaphysis. The narrow transverse septa between the cells and part of the longitudinal trabeculae between the cell columns are resorbed, while capillaries and connective tissue invade the lacunae left by the hypertrophic cells. According to Dodds (1932), chondroblasts as well as blood vessel endothelium and connective tissue cells take part in this resorptive process. The calcified



(*a*) MICRORADIOGRAM 10–50 Å OF DECALCIFIED 5-μ THICK MICROTOME SECTION OF A TOOTH FROM A RACHITIC DOG

The predentine zone is wider than normal; both this area and the interglobular dentine are visible as darker areas, indicating a lower mass per unit area for both predentine and interglobular dentine (× 100).



(*b*) MICRORADIOGRAM 10–50 Å OF A 5-μ THICK DECALCIFIED MICROTOME SECTION OF A TOOTH FROM RACHITIC DOG

The picture is taken fairly high up in the tooth and shows pulp, odontoblasts and the predentine area, which is somewhat widened and shows less absorption than the "mineralized" dentine. A somewhat lower degree of absorption is also seen in a wide zone of the dentine parallel with the predentine zone and fairly clearly delineated from earlier-formed dentine. No evidence of structural changes in the form of interglobular dentine in this zone (× 192).

follows. The thickened cartilage is invaded by large vascular tufts; the normal process with capillaries penetrating into each successive cell in a cell column has usually disappeared. Calcification is absent in smaller or larger parts of the zone of provisional calcification, and, in some cases, has ceased in the entire area. The first observations about this fact were also published by the last-mentioned authors. According to Kölliker (1847, 1850), Meyer (1849), and Virchow (1853), however, they had also observed calcification of the cartilage cells, an idea which was dispelled by Müller's publication in 1858. The cartilage matrix projecting downwards into the metaphysis contains calcium-salts in some parts. The organization of this cartilage matrix, which is incompletely or not at all calcified, is extremely irregular; its normal, even appearance is lost. On these irregularly organized parts of the cartilage matrix excessive amounts of osteoid are present, due to defective calcification of this tissue after it has been deposited by the osteoblasts. Osteoid is distributed with extreme irregularity in the metaphysis. It is often seen on both sides of a trabecula, in other cases it is found on only one side, while the other side shows calcified bone tissue which is being removed by osteoclasts. In some places, seams of osteoid tissue adjoin areas with calcified bone (Park, 1938–39). Abnormally large masses of osteoid are commonly observed in cases of vitamin-D deficiency. As pointed out by Pommer (1885), Schmorl (1909) and Park (1938–39), a superabundance of osteoid tissue may be the sole indication of a rachitic disease in older children, because bone growth at that age has slowed down and the epiphyseal plate, presumably as a result of this, is calcified on a normal scale. In adults the presence of osteoid masses is likewise the only sign of an osteomalacic disease, because in this case length growth has been completed and the epiphyseal plate is closed. On the other hand, cases of rickets do occur in which, despite apparent rachitic changes in the growth zone, no abnormal amounts of osteoid can be seen. This is caused by the osteoblasts ceasing to function, while the cartilage cells, on the other hand, have continued their division and growth. This condition has been particularly stressed by Park (1938–39).

Dodds and Cameron (1939) and Park (1938–39) have described in detail the rachitic changes in the metaphyseal cartilage as they occur in rats and man. Park (1938–39) observed that the cartilage within certain areas is changed in such fashion that it assumes an increasing resemblance to osteoid. The matrix loses its stainability, while the appearance of the cartilage cells undergoes several evolutions, some of them finally assuming the appearance of bone cells. Park (1938–39) gave this tissue the name of pseudo-osteoid; he considered these changes due to degenerative processes in the cartilage rather than to metaplasia of cartilage into bone. Dodds and Cameron (1939), on the other hand, concluded from their investigations that cartilage cells in uninvaded lacunae form a tissue which, they considered, was most closely designated by the name osteoid, since it resembles bone rather than cartilage. They regarded this as a metaplastic process.

As has been discussed earlier, the rachitic organism is unable to manage the mineralization of the zone of provisional calcification on a normal scale. This defective calcification of the growth zone has been mentioned by several authors as the first appreciable change in rickets (among others Schmorl, 1909; Dodds and Cameron, 1934; Park, 1938–39).

Schmorl (1909) regarded defective calcification of the zone of provisional calcification as the first rachitic change. According to this author, these defects have a focal distribution, and because of this focal impairment the capillaries are directed into the cartilage along a different course. The areas in the cartilage where calcification has disappeared are easier to penetrate for the vascular tufts, whereas capillary invasion of the cartilage is normal in the places where calcium has been deposited between the cells. Once the vessels have penetrated into the cartilage, they increase in size. Because these large vessels require a considerable part of the available blood supply at the epiphyseal plate, the capillaries in the normally calcified areas are drained of blood. As a consequence of the impaired circulation, the cartilage in these areas is not adequately resorbed. Thus, the cartilage grows thicker, first in these areas but, later, also in places where calcium deposition has not taken place, due to the fact that the vascular tufts penetrate deeply into the cartilage but have only a moderate resorptive effect within limited areas.

Both Shohl and Wohlbach (1936) and Wohlbach and Bessey (1942) regarded the absence of the zone with "clear cartilage cells," normally seen over the capillaries, as the first morphologic change to become apparent in rickets. In a later paper, Park (1954*a*, *b*) also stressed the absence of the zone containing clear, empty or partially-empty cartilage cell lacunae as indicating an early rachitic lesion. Shohl and Wohlbach (1936), Wohlbach and Bessey (1942), and Park (1954*b*) all ascribed this phenomenon to inability of the cartilage cells to complete their normal cytomorphic cycle. Simultaneously, or immediately afterwards, calcification of the zone of provisional calcification begins to deteriorate. As the cartilage cells fail to become vacuolated on a normal scale, the cartilage becomes inaccessible for penetration by capillaries and osteoblasts and, consequently, becomes thickened. According to Park (1938–39, 1954*b*), the defective

longitudinal cartilage walls become the site of bone deposition by osteoblasts developed from the connective tissue cells of the marrow. The calcified cartilage provides a solid foundation upon which bone may be formed. Through this process, bone and cartilage become firmly united, preventing the epiphysis from separating from the diaphysis or from slipping sideways in relation to it. It is generally assumed, furthermore, that the capillaries are guided into penetrating the cartilage cells by the fact that calcification exclusively, or at least predominantly, occurs in the longitudinal trabeculae of the cartilage matrix.

The part of the cartilage composed of these calcified longitudinal trabeculae, as well as the adjacent bone trabeculae, is known as the primary spongiosa. Farther down towards the diaphysis, the structure of the calcified matrix, with the newly formed bone, is being significantly altered, due to osteoclastic resorption of certain parts of the bone trabeculae and the enclosed cartilage. Concurrently, the deposition of new bone tissue on the remaining parts of the primary spongiosa continues. As a result of these processes, the number of bone trabeculae is reduced, but those that are not resorbed, increase in size. This region is called the secondary spongiosa.

In Pommer's works (1885, 1925) it was emphasized that the process of endochondral ossification is identical with all bone formation, in that the osteoblasts first form an organic tissue, osteoid, which subsequently becomes calcified and develops into bone. Consequently, seams of osteoid tissue are found between the osteoblasts and the calcified bone tissue. Similar observations were made by Müller (1858), Wieland (1909), and Erdheim (1914). Weidenreich (1923), on the other hand, believed these osteoid seams to be an artifact; whereas Freudenberg and György (1923) and Mass (1923) saw osteogenesis as a process where deposit of calcium salts in the tissue occurs simultaneously with its formation by the osteoblasts. Under normal conditions only extremely narrow seams of osteoid can therefore be seen. McLean and Bloom (1940) have studied this problem in more detail. They concluded from their investigations that, in the normal endochondral ossification process, the greater part of the newly-formed bone tissue, as a rule, becomes calcified either at the time the organic matrix is formed, or so soon afterwards that any intermediate stage of osteoid tissue cannot be observed. They consequently suggested that so-called physiological osteoid is not an indispensable phase of osteogenesis. They admitted that it might sometimes be seen even under apparently normal conditions, but they attributed this, in general, to local defects in the supply of bone salts. Although the various authors have differing opinions concerning osteoid as a necessary link in the normal ossification process, it is generally agreed that the amount of osteoid under normal conditions is very small and that it collects in narrow seams.

From the preceding account it is evident that endochondral ossification consists of a co-ordinated sequence of cellular processes. The initial divisions, enlargement, and degeneration of cartilage cells in the growth zone is followed by an invasion of capillaries and connective tissue cells from the diaphysis, and by osteoblastic formation of bone tissue on the part of the cartilage matrix that has not been resorbed. As the final process, structural changes take place in the new-born tissue. As a result of these processes, an increase in the length of the bone occurs.

2. The Growth Zone in Vitamin-D Deficiency States

The rachitic alterations in the growth zone that become visible on histological examination have been studied by numerous authors. Early studies on human material were made by Kölliker (1847, 1850), Meyer (1849), Virchow (1853), Müller (1858). Pommer (1885, 1925) and Schmorl (1909) contributed further careful descriptions; in stressing the significance of osteoid in the rachitic bone tissue these authors have added considerably to medical knowledge. In 1914, Erdheim published a detailed description of spontaneous rickets in rats. Successful attempts to produce rickets experimentally, and recognition of rickets as a vitamin-D deficiency disease have both been instrumental in creating new possibilities for study of the pathological tissue changes. Among the reports of this type of investigation, mention should be made of those presented by McCollum *et al.* (1921*b*), Mellanby (1921), Pappenheimer (1922), Shohl and Wohlbach (1936), Nicolaysen and Jansen (1939), and Dodds and Cameron (1934, 1938, 1939, 1943). A most instructive account has been published by Park (1938–39); some later papers by this author are also concerned with rickets (1954*a*, *b*).

In rickets, the growth zone is found to be thickened and there is a significant predominance of the hypertrophic cells, a condition which was recognized at an early stage by Kölliker (1847, 1850), Meyer (1849), Virchow (1853), and Müller (1858). In addition, the normal columnar arrangement is, in many cases, no longer apparent. Instead of the normally sharp and regular outline at the diaphysis, there is an uneven border between cartilage, and bone with cartilage extensions of varying length projecting into the metaphysis (Plate V*A* (*b*)). The cartilage projecting into the metaphysis contains altered cells of varied appearance, which is described in more detail as

(1909) from human material. Dodds and Cameron (1938) and Bailie and Irving (1948), on the other hand, stated that mineralization of the cartilage on healing is first resumed in the longitudinal cartilage matrix in the border zone between epiphyseal plate and metaphysis, and, in particular, in the area adjoining the cortex of the bone. According to these authors, calcification as described by Müller (1858) and Schmorl (1909) occurs as a later phase in the healing process. When the cartilage has become calcified, it is invaded by capillaries which partly come laterally from the cortex, partly distally from the diaphysis (Dodds and Cameron, 1938). Through the resultant resorption of cartilage, the border between epiphysis and metaphysis is eventually restored to its normal appearance.

Simultaneously with mineralization and reorganization of the rachitic cartilaginous tissue, healing processes are also initiated in the remaining tissues of metaphysis and bone.

According to most authors, calcification of the osteoid tissue runs parallel with mineralization of the cartilage. The superfluous part of the calcified bone tissue is resorbed. In the rachitic metaphysis, according to Dodds and Cameron (1938), resorption starts at the border of the cartilage and spreads down to the diaphysis. On the cartilage trabeculae at the epiphyseal plate, which gradually resumes its normal function, the osteoblasts deposit normal bone tissue. As the healing process advances, the bone gradually resumes its normal gross and microscopic appearance. Eventually, the rachitic changes completely disappear and the growth zone is restored to normal.

If healing is interrupted before normal conditions have been completely restored, resulting in renewed progress of the rachitic disease, the cartilage which has become calcified during the period of healing is, according to Schmorl (1909), shifted down to the diaphysis. Over this line a zone of uncalcified cartilage is seen in such cases. If healing is resumed, mineral salts are again deposited in the cartilage at the beginning of the hypertrophic zone. Consequently, two separate lines of mineralization can be seen to enclose a zone of uncalcified cartilage. The distance between these lines gives an approximate idea of the duration of the relapse into the rachitic disease (Schmorl, 1909). Bailie and Irving (1948) observed that, in experimental rickets in rats, the tissues calcified during the period of healing become separated from the epiphysis, and gradually move down in the metaphysis as a result of both marrow and osteoid formation and continuous cartilage removal in the zone above these calcified tissues.

Calcification of cartilage and osteoid tissue has been observed in chronic rickets, even though there was no question of an actual regression of the disease (Hess, 1930; Park, 1938–39; Follis *et al.*, 1952; Engfeldt and Zetterström, 1955). Minor calcification of rachitic tissues should therefore not be interpreted as a definite indication that healing processes are taking place.

III. BIOPHYSICAL INVESTIGATIONS

A. Introduction

Investigations carried out with traditional histological research methods have supplied information about cell and tissue changes in cartilage and bone. Since rickets is, to a great extent, characterized by disturbances in the calcium supply to tissues which under normal circumstances would become calcified, the investigations have, in many instances, been concentrated on defining the nature of these disturbances. The distribution of mineral salts has been studied both in completely or partially decalcified, and in undecalcified sections. Earlier investigations were, as a rule, made on partially or completely decalcified sections, stained with haematoxylin or alizarin; in other studies, undecalcified sections were used, the mineral salts being identified by staining with silver nitrate, haematoxylin, or alizarin (McLean and Bloom, 1940; Freeman and McLean, 1941). The validity of the results of studies made on completely or partially decalcified sections, in which certain tinctorial variations in the organic substance were adopted as a criterion of the distribution of mineral salts, is open to question. In undecalcified sections the mineral salts are conserved in the tissues, which admittedly permits a direct study of the mineralization in such specimens, but the histological staining methods employed in this type of investigation are not specific. Deposit of silver in certain tissue structures does not specifically imply the presence of calcium salts. As to alizarin, this substance often does not stain older calcium deposits, whereas the alleged staining reaction of hematoxylin to calcium often fails to materialize (Cameron, 1930). Furthermore, the histological methods used in earlier investigations do not permit a quantitative evaluation of the mineral salts present in calcified tissue.

During the past decade, new biophysical research methods have become available. These make it possible to obtain accurate, and to some extent also quantitative, information concerning the processes of mineralization in bone tissue. These methods include microradiography, autoradiography, and X-ray diffraction.

calcification of the cartilage leads to compression of part of the longitudinal trabeculae and surrounding cartilage tissue. As the cartilage matrix between some of the cell columns in the zone of provisional calcification does not become adequately calcified, the cell columns are no longer individually invaded by a capillary; instead, several columns are attacked by a single large vessel. This is a highly characteristic indication of the disease. Since both the inability of the cells to complete their cycle and the impairment in calcification do not progress at an equal rate everywhere, the capillaries are forced to seek their way into the cartilage at the point of least resistance. The vessels will consequently search their way along the border of epiphysis and metaphysis until they find a vulnerable spot. This, according to these authors, explains the irregular vascular invasion of the cartilage in rickets.

The thickening of the cartilage plate attracted attention at an early stage. Virchow (1853) considered it due to increased production of cartilage tissue. Schmorl (1909), on the other hand, rejected this theory and ascribed the thickening of the cartilage to inadequate resorption of the normal quantities of cartilage produced. This theory received strong support in Dodds and Cameron's scrupulous histological studies of the growth zone in rachitic rats (1934). They agreed with several earlier authors in observing that the thickening is mainly caused by lengthening of the zone containing the largest cells in the epiphyseal plate. Above this zone, mitosis and production of cells occurs on a normal scale or, in advanced cases, on a reduced scale. The increased thickness of the cartilage must consequently be seen as the result of defective resorption and not of increased production of cartilage tissue. The preponderance of rickets' researchers adhere to the same theory, among them Park (1938–39). Other factors mentioned in substantiation of this theory are the retarded length growth of the cylindrical bones in rickets (Schmorl, 1909; Dodds and Cameron, 1943) and the fact that widening of the cartilage area is most conspicuous in the bones which normally would undergo the most rapid growth (Schmorl, 1909).

Summing up, it can be said, consequently, that the morphological alterations characterizing the growth zone in rickets are the following:

1. Impaired or discontinued calcification of the zone of provisional calcification, and of the persisting portions of cartilage matrix projecting into the metaphysis.

2. Increase of the number of hypertrophic cells and thickening of the epiphyseal cartilage.

3. Irregular invasion of the cartilage by large vessels, and irregularity of the border between bone and cartilage, with tongues of altered cartilage extending into the metaphysis.

4. Excessive osteoid deposits on the cartilage trabeculae.

E. Healing of Lesions

The rachitic lesions in man, as they appear during the healing process, were thoroughly studied at an early stage by Müller (1858) and Schmorl (1909). Research has since then been supplemented by *i.a.* Park (1938–39), Follis *et al.* (1952). Successful attempts to produce rickets in rats offered new possibilities for study of the healing process, as bone tissue could be taken from the animals and investigated at various stages after healing had begun. Among the studies of this type, mention should be made of those performed by Shipley *et al.* (1920–21*b*), Pappenheimer (1922), Dodds and Cameron (1938), and Bailie and Irving (1948).

The healing processes can roughly be divided into those occurring in cartilage and those taking place in bone. As mentioned in the preceding account, Shohl and Wohlbach (1936) and Wohlbach and Bessey (1942) regarded, as the earliest rachitic change, absence of the zone of clear cells which normally lies over the capillaries at the border of bone and cartilage. These authors stated that the first sign of healing, similarly, is the return of this zone of clear cells. They reported to have observed this phenomenon as early as 24 hours after healing had been induced. Following this, the cells are penetrated by vast numbers of capillaries, and calcification of the surrounding cartilage matrix occurs. Other authors reported calcification of cartilage and bone tissue as the first process to become apparent. Müller (1858) and Schmorl (1909) were among the first to point out that this calcification does not take place at the border of bone and cartilage, but in the matrix which separates the cell columns, somewhat higher up in the growth zone. According to Müller (1858), calcification is, during healing, initiated in the area where it would normally have started in the absence of rickets, *i.e.* in the matrix between the younger hypertrophic cells. From this area, calcification spreads down to the primary spongosia in the metaphysis. At the border of bone and cartilage, these authors, among other things, observed that the matrix between the last couple of cells, in cases of chronic rickets, may be spottily calcified. Müller (1858) and Schmorl (1909) interpreted this phenomenon as remnants of the normal mineralization achieved before the rachitic changes manifested themselves. Pappenheimer (1922) and Shipley *et al.* (1920–21*b*) observed, in rachitic rats, a distribution of calcium salts in the healing cartilage, similar to that reported by Müller (1858) and Schmorl

are, instead, tangentially-sectioned longitudinal matrix trabeculae which normally do become calcified.

On the trabeculae in the primary spongiosa, osteoid seams are found on an extremely small scale. Farther down towards the secondary spongiosa, they increase somewhat in number and in width. These results are in close agreement with those reported by McLean and Bloom (1940). The presence of osteoid in isolated spots should be interpreted as an indication that osteogenesis is initiated by the development of an organic tissue, which subsequently becomes calcified. In the area where osteoid cannot be observed, mineralization of the organic tissue has progressed so rapidly that a separate phase of osteoid cannot be demonstrated in the specimen.

Studies of the organic component of epiphyseal cartilage have shown that the dry weight of the cartilage matrix in the resting cartilage is relatively high and, on comparison with the proliferation zone and the zone of hypertrophic cells, the dry weight of the matrix in the latter regions is often found to be somewhat lower. In other cases it is impossible to find any significant difference in dry weight per unit area of matrix between these zones. The dry weight per unit area of the cells in the epiphyseal plate seems to increase as they proceed from the resting to the proliferation stage, only to decrease after that, due to vacuolation of the cells and disintegration of the nuclei (Plate V*B* (*a*)). Freeman (1956), who also made a study of these problems, using microradiographic methods, considered the mass of organic substance per unit area to be larger in the cartilage matrix in the zone of provisional calcification than in the area just above it. In many cases we were able to make the same observation, but a detailed study of this problem has shown these results to be of doubtful significance. The fact is that, adjoining the hypertrophic cells, the matrix contains a narrow zone which has a higher dry weight per unit area than the central parts of the longitudinal septa. During the cytomorphic cycle the cell columns gradually approach each other towards the metaphysis, with the result that the matrix between the columns is narrowed. In the longitudinal matrix septa in the epiphyseal cartilage, as has been mentioned in the foregoing, narrow areas with a high dry weight are found adjoining the cartilage lacunae just over, and, in particular, within the zone of provisional calcification. These areas will finally touch each other as the longitudinal septa become narrower. This is most often seen in the zone of provisional calcification. Because of this, the narrow longitudinal septa which then develop have a higher mass per unit area than the central parts of the matrix higher up in the growth zone. The higher dry weight found around the hypertrophic cartilage cell lacunae may, therefore, very well be regarded as a consequence of the increase in volume of the lacunae during cytomorphosis, since this implies compression of the surrounding matrix and, consequently, increase of the dry weight per unit area (Plate VI*A* (*a*)). The same observation with respect to dry weight applies to the transverse septa high up in the hypertrophic zone, and in the zone of provisional calcification. On mineralization of the cartilage, therefore, deposition of calcium salts does not with any certainty imply a simultaneous increase in dry weight of the organic component. In the primary spongiosa the cartilage trabeculae are covered with bone tissue, the organic component of which has a considerably higher mass per unit area than the cartilage.

2. The Growth Zone in Vitamin-D Deficiency States

Using the same procedure as mentioned above, pathological changes in the growth zone have been investigated in dogs affected by experimentally-produced rickets. It was found that in mild rickets the zone of provisional calcification is incompletely calcified. This implies that this zone contains large areas where the longitudinal trabeculae have not become calcified. In other areas, although calcium has been deposited in the matrix, the amount of mineral salts is small. In more advanced cases of rickets, where the cartilage plate has become considerably widened, the zone of provisional calcification is generally not calcified. Even in rickets of long standing, however, isolated areas of calcium deposit may be seen in the matrix between the hypertrophic cells or in the part of the matrix corresponding to the zone of provisional calcification (Plate V*B* (*b*)). The rachitic metaphysis contains areas of uncalcified cartilage as well as areas of calcified cartilage. The latter are often composed of broad tongues of cartilage matrix, and cells in which the matrix between the cell columns has been calcified. This calcification is often irregularly distributed in the cartilage matrix. Sometimes, mineral salts are observed in the matrix only within a small area of the otherwise uncalcified cartilage, whereas in other cases the matrix between the cell columns in these cartilage projections is found to be almost entirely calcified. Often, the altered cartilage described by Park (1938–39), among others, which has been called pseudo-osteoid, also contains deposits of calcium salts. Dodds and Cameron define the altered tissue which may be seen in uninvaded cartilage cell lacunae, as osteoid; in many cases these altered areas are also calcified (Plate VI*B*).

In the rachitic specimens, the trabeculae in the metaphysis are irregularly organized (Plates VI*A* (*b*) and VII*A* cf. IX*A* (*a*)). Some of them consist of more or

The technique of microradiography makes it possible to study the quantity and distribution of mineral salts in bone tissue (Engström, 1955), since at a roentgen radiation of 1–3 Å absorption of roentgen rays in the hard tissues is almost exclusively due to the mineral salts (Engfeldt and Engström, 1954). For investigations of this type, the material may be either undecalcified ground sections or undecalcified microtome sections. With roentgen radiation of considerably longer wavelengths (10–50 Å), the microradiographic procedure may be used to determine the dry weight per unit area of cells and cell components (Engström and Lindström, 1958). In consequence of applying this technique on decalcified sections, knowledge is gained concerning the dry weight per unit area of the organic component, whereas a study of undecalcified sections containing mineral salts within certain structures also provides information about the mineralization.

Another method for determining the dry weight per unit area is microinterferometry. Davies and Wilkins (1952), Barer (1953) and Davies (1958) described the theoretical basis for this technique, which they employed in different investigations. Microinterferometry may also be useful in studies of bone tissue. With this method, the dry weight per unit area can be determined for both the organic and the inorganic component. To this end, the same sections are studied with the interferometry microscope before and after decalcification, after which the amount of calcium salts in the tissue can be calculated. There are consequently two independent techniques which may be used to determine the mass of the inorganic and organic components of bone tissue. Comparative studies with both methods have shown good correlation (Davies and Engström, 1954).

Autoradiographic investigations with radiocalcium and phosphorus-labelled sodium phosphate can supply valuable information concerning growth and metabolism in the mineralized part of the bone tissue. With the aid of sulphur-labelled sodium sulphate information about the organic component of bone tissue may be elicited.

The nature of the mineral salts, their crystalline structure, as well as size and orientation of the crystallites may be studied with various X-ray diffraction methods (Carlström, 1955). Microscopic examination in polarized light, to conclude, may be used to ascertain *i.a.* how the collagen is distributed and oriented in the bone tissue.

The biophysical methods described in the foregoing have been employed in numerous studies of normal and pathologically changed bone tissue. (For references *see* Engfeldt, 1958). For the present account, it is of interest that these methods, in combination with traditional histological techniques, have also been used in studies of experimentally-produced rachitic changes in the bone tissue of dogs. In this way, biophysical information has been added to present knowledge of the disturbances in mineralization apparent in rachitic bone tissue. We intend, in the following pages, to review the observations made in our laboratory during such studies. The results of these investigations have, in part, been published previously (Engfeldt and Zetterström, 1955), whereas part of the material is in preparation and will be reported in detail elsewhere. The histological aspect of normal and rachitic growth zones has been ascribed in the foregoing. It should have become clear that different opinions prevail with regard to the mineralization of the growth zone.

B. Microradiographic Studies

1. The Normal Growth Zone

Our investigations have been concerned with the mineralization of cartilage matrix and bone trabeculae *in the metaphysis adjoining the epiphyseal cartilage.* These structures were studied both microradiographically and by microinterferometry. The investigations of normal growth zones in our material revealed that mineralization of the zone of provisional calcification occurs in the longitudinal matrix trabeculae that lie between the cell columns. Calcification starts close to the cells and progresses into the matrix between the cell columns. It is extremely rare, under normal conditions, to find uncalcified matrix between the cell columns. Where this is observed, the longitudinal matrix septa are extremely narrow. The mineralization stretches, as a rule, 3–4 cells ahead of the capillaries, although the normal material shows variations in this respect. In some cases mineralization is only one cell, in others as much as 5–7 cells in advance (Plate V*B* (*a*)). On comparison between the mineralization pattern at the border of bone and cartilage in ribs, and that apparent in the metacarpal and metatarsal bones, mineralization in the latter is seen to extend beyond a greater number of cells. The cartilage matrix between the individual cells in the cell columns (transverse septa) in the provisional calcification zone are as a rule not calcified, but exceptions to this rule do occur in isolated spots. In such areas the transverse septa are in many instances remarkably wide. Because of the irregular wavy outline of the cell columns, the matrix separating them will, to some extent, project between the cells. In some cases, where a longitudinal section happens to go through just this area, the resulting pattern may be mistaken for that described above with respect to the transverse septa. In such cases, however, there is no question of calcified transverse bars; these structures

of low or no uptake gradually develops below the cartilage as growth proceeds. In the compact bone, the uptake is high in osteones with a low content of mineral salts. In the highly mineralized osteones, on the other hand, the uptake is extremely low, whereas there is practically no uptake at all in the walls of the resorption cavities (Engfeldt *et al.*, 1952). Uptake of these isotopes in bone tissue is a very rapid process and occurs predominantly in areas with a low content of mineral salts, which are newly formed and still undergoing mineralization. *In vitro* studies with radiocalcium or radiophosphate produce a similar pattern of uptake as that observed after *in vivo* administration of the isotopes. It may be assumed, therefore, that uptake of the radioactive isotopes is due, in part, to new deposition of mineral salts, partly also to ionic exchange. The latter process occurs predominantly in low mineralized areas.

Autoradiographic studies with radiocalcium or radioactive phosphate show that, in rickets, the unlabelled zone corresponding to the epiphyseal cartilage, after a single injection, is wider than under normal conditions. The uptake in the zone below the epiphyseal cartilage appears to be less marked than in normal cases, due to the fact that in rickets the amount of mineralized tissue that can be labelled is reduced. The individual mineralized structures, on the other hand, are labelled on a normal scale. The pattern of uptake, however, is highly irregular, due to the disorganization of the trabeculae in rickets. In the compact bone, the uptake of radioactivity corresponds to young Haversian systems with a low content of mineral salts, whereas the uptake in older, highly mineralized systems is low. The unmineralized osteoid tissue shows no uptake, but the areas in this tissue where mineral salts can be demonstrated are clearly labelled.

Summing up the results of our autoradiographic studies in short-time experiments with radiocalcium or radiophosphate, it may be concluded that in rickets the uptake within individual structures is of the same order of magnitude as under normal conditions. The uptake is furthermore seen in the same structures that are labelled normally. These observations may seem surprising, but may probably be explained as follows. In rickets the calcification process is disturbed, with the result that newly-formed bone tissue is poorly or not at all calcified. In addition, mineralization spreads in the growing Haversian systems at a slower rate than under normal conditions. Because of these defects, relatively large amounts of lowly mineralized bone tissue develop. It has been shown that in structures of this type, in the bone tissue, an extensive exchange of ions takes place with the surrounding body fluid. The uptake of radioactive isotopes in bone tissue in rickets appears, consequently, to be of the same order of magnitude as under normal conditions, despite the fact that new deposition of mineralized tissues is greatly reduced in rickets. The observation that the first rapid uptake of radioactive isotopes, due to ionic exchange, is not appreciably changed in rickets, has been made earlier by Copp *et al.* (1951) and Claassen and Wöstmann (1953). In long-term experiments, however, the uptake of radiocalcium or radiophosphate in bone tissue becomes less than under normal conditions, due to reduced deposition of new bone salts (Copp *et al.*, 1951; Engfeldt and Zetterström, 1955).

O

From the chemical and autoradiographic investigations with sulphur-labelled sodium sulphate it is known that the isotope is absorbed by several tissues in the animal organism after administration of a single dose. One of these tissues is cartilage (Dziewiatkowskic *et al.*, 1949; Dziewiatkowskic, 1951*a*; Boström and Odeblad, 1953; Belanger, 1954). With respect to this tissue, Dziewiatkowskic (1951*b*) and Boström (1953) were able to demonstrate that the injected radiosulphate is incorporated in the chondroitin sulphate, synthetized in the cartilage. Labelling is also seen in bone tissue after administration of radiosulphate (Dziewiatkowskic, 1951*a*). Radiosulphate is absorbed both in the organic and in the inorganic component of this tissue (Engfeldt *et al.*, 1954; Engfeldt and Hjertquist, 1955). It has been assumed that incorporation of radiosulphate in the organic part of the bone tissue is due to its mucopolysaccharide component. The uptake in the inorganic part is probably due to heterogeneous ionic exchange between bone salt and surrounding body fluid (Engfeldt and Hjertquist, 1955).

Dziewiatkowskic (1954) has made a study of sulphate metabolism in rickets. Combined autoradiographic and chemical studies on rachitic rats injected with radiosulphate showed the rate of synthesis of chondroitin sulphate in the growth zone and adjoining bone tissue to be as high as in normal animals. After the rachitic rats had been given vitamin D_2, he observed an increase in the rate of synthesis of chondroitin sulphate in the skeleton. This author concluded from his investigations that in rachitic rats chondroitin sulphate is poorly utilized, and that vitamin D_2 is capable of accelerating the process.

In our laboratory, autoradiographic studies with radiosulphate have been made of growth zones from normal and rachitic puppies. The material was taken from these animals three days after they had been given a single dose of radiosulphate. Decalcified and undecalcified sections from the growth zones in ribs and long bones of normal animals produce autoradiograms which show a marked uptake of radio-

less mineralized cartilage on which, in some cases, mineralized bone or osteoid tissue has been deposited. Other trabeculae consist exclusively of bone and osteoid (Plate VII*B*). Osteoid tissue is present in varying amounts, and in seams of different width. Because of this, the mineralization of the trabeculae shows a highly irregular pattern. Some parts are highly mineralized, whereas the amount of mineral salts is small in other areas. Some trabeculae contain entirely uncalcified areas, representing remnants of cartilage cells.

With respect to the organic component in the rachitic cartilage it can be stated that, similar to normal conditions, the dry weight per unit area is higher for the cells in the proliferation zone than in the resting stage, and, subsequently, decreases in the zone of hypertrophic cells.

The dry weight per unit area of cartilage matrix is, furthermore, similar to what has been observed in the normal cases, that is as high as or higher in the resting cartilage in comparison with the proliferation zone. In the zone of hypertrophic cells, the same conditions prevail as described for normal specimens. The matrix in the tongues of cartilage projecting into the rachitic metaphysis varies with respect to dry weight. The matrix within certain of these areas has a lower dry weight than the matrix above the irregular border between cartilage and bone. In rickets, as in normal cases, the dry weight of the uncalcified cartilage matrix is lower than in the corresponding structures of bone or osteoid (Plate VI*A* (*b*)).

3. The Normal Compact Bone Tissue

It is known, from microradiographic investigations that the different structures in the compact bone of normal long bones do not become uniformly calcified (Amprino and Engström, 1952). The outer and inner circumferential lamellae, as well as the interstitial lamellae, are, as a rule, evenly and highly mineralized. The mineralization of the osteones, on the other hand, shows variations. Young osteones are poorly mineralized, but this improves as they become older. Within the individual osteone, the lowest degree of mineralization is initially found at the periphery, whereas mineralization increases towards the Haversian canal. In older osteones, the mineral salts are evenly distributed over the entire structure. Adjoining the Haversian canal lies a narrow zone of very poorly mineralized, or entirely unmineralized tissue, containing osteocytes. This zone is metachromatic and has a positive PAS reaction (Engfeldt and Hjertquist, 1955). In the osteogenetic process, this tissue is first deposited by osteoblasts, and subsequently becomes mineralized. In normal bone, the collagen is arranged in a regular pattern; in the osteones it is deposited concentrically around the Haversian canal.

4. The Compact Bone Tissue in Vitamin-D Deficiency States

The compact bone in rachitic puppies similarly shows varying degrees of mineralization. Large resorption cavities are found within certain areas. The walls of these cavities consist of bone tissue of varying mineralization. At the same time, highly mineralized osteones can be observed, in which the collagen fibres are arranged in a normal pattern. These osteones have been formed before the rachitic disease developed. Adjoining the Haversian canal the osteones generally have a narrow border of uncalcified tissue with regularly arranged collagen fibres. This zone is well defined from the mineralized part of the osteone, and from the osteoid tissue within the canal. This osteoid tissue contains a varying amount of collagen fibres; in some parts the collagen content is high, in others low. Instead of concentric bundles of collagen, this tissue contains short, broad bundles of collagen fibres running perpendicular to the long axis of the canal. The osteoid tissue is mineralized either not at all, or very poorly (Plate VIII). It is very common, however, to find an extremely thin mineralized zone adjoining the soft tissue of the Haversian canal. The same condition described in the foregoing may also be observed in bone adjoining the periosteum.

C. *Autoradiographic Studies*

In addition to our other investigations, autoradiographic studies with radiocalcium and radioactive phosphate have been made on bone tissue from rachitic animals. With these isotopes as tracers, we intended to study the process of mineralization in rickets. To outline the background against which the observations in rickets must be seen in order to be properly evaluated, a short description of the autoradiographic observations with respect to the uptake of radiocalcium and radioactive phosphate in bone tissue from normal animals, reported by numerous authors, is given here. Autoradiographic examination of the growth zone in normal animals, injected *in vivo* with radiocalcium or radiophosphate, shows that the uptake of radioactivity in the cartilage is restricted to the zone where provisional calcification takes place (Plate IX*A*). Below the cartilage, a zone of high uptake is found corresponding to the primary and secondary spongiosa (Engfeldt and Hjertquist, 1954). In the autoradiogram, this labelling is seen to correspond to the regular pattern of the bone trabeculae. Since the available amount of a radioactive isotope in the blood rapidly decreases after a single injection, a zone

capillary invasion and cartilage resorption, as the invasion by capillaries is preceded by deposition of mineral salts in the longitudinal septa.

IV. TISSUE CHANGES IN RICKETS OF ATYPICAL GENESIS

In the preceding pages, a description has been given of the changes which occur in the growing bone tissue in cases of vitamin-D deficiency. Similar changes may manifest themselves under different conditions. Accordingly, a number of different pathological conditions may, under certain circumstances, be complicated by changes in the hard tissues suggestive of rickets. A common feature of these defects is that they either involve deviations in the metabolism of mineral salts, or anomalous development of the matrix which prevents it from becoming calcified in the normal manner. These features are present where bone-forming ions or vitamin D are not resorbed from the intestine in sufficient quantities, as for instance in steatorrhea. Some diseases of the liver may likewise be complicated by inability to resorb vitamin D on a normal scale. Chronic diseases of the kidney, involving glomerular or tubular insufficiency, are sometimes complicated by similar disturbances of the metabolism of mineral salts, which may result in changes resembling those seen in rickets. The aetiologic relation between basic disorder and manifestation of rachitic alterations is obvious in the aforementioned conditions. Exceptional cases do occur, however, where the primary cause of the alterations in the hard tissues is obscure. To this category belongs, among others, so-called primary vitamin-D-resistant rickets, which is characterized by changes in the growth zones of the bone tissue similar to those seen in genuine rickets. This disease varies from genuine rickets in that enormous doses of vitamin D are required for increased mineralization of bone tissue. This, however, does not effect actual healing. The disease is of genetic origin and disappears spontaneously at puberty. The histological changes in the epiphyseal and metaphyseal parts of the bone tissue do not vary from those observed in ordinary vitamin-D-deficiency rickets. The important difference is that the structure of the bone tissue, and in particular of the compact bone, is seriously disorganized; poorly-mineralized areas are scattered through zones of high mineralization, resulting in a mosaic pattern suggestive of that seen in Paget's disease of bone. A characteristic feature is that, in some areas, the lacunae of the bone tissue are surrounded by a larger zone which is poorly mineralized, or where mineralization is entirely lacking. These structural changes in the bone tissue persist even under intensive vitamin-D treatment, although this, as can be seen on roentgenographic examination, does succeed in increasing the calcium content of the skeleton (Engfeldt *et al.*, 1956). One more disease, in which rachitic changes in the growth zone are in evidence, deserves mention, viz. osteodysmetamorphosis foetalis, because of the theoretical interest attached to the condition. In this disease, which also is of genetic origin, extremely low values are found for alkaline phosphatase activity in tissues or serum. Disturbances in the growing skeleton are accompanied by severe morphological changes, reflected, among other things, by absence of or defects in the mineralization of the matrix (Engfeldt and Zetterström, 1954). In the cases we have observed, hypercalcemia and nephrocalcinosis were present, and a hypothesis has been advanced that the disease is caused by hypersensitivity to vitamin D.

V. OSTEOMALACIA

The preceding account has been chiefly concerned with the changes which occur in the growing organism as a result of vitamin-D deficiency. In adults, vitamin-D deficiency can affect only the regenerative processes in the bone tissue, since endochondral bone growth has been completed. In such cases, vitamin-D deficiency implies a decrease in the amount of newly-formed mineralized bone tissue, and an increase in the quantity of osteoid tissue which obliterates the bone trabeculae. This condition, known as osteomalacia, may lead to serious skeletal deformities as the mechanical resistance of the bone tissue decreases. Osteomalacia due to vitamin-D deficiency is, nowadays, hardly a prevalent disease in civilized countries with a high standard of living. On the other hand, the disease is sometimes encountered in China and in India, in particular, in women whose mineral metabolism has been put under strain by repeated pregnancies (Snapper, 1957). The cases of osteomalacia occurring in civilized countries are almost exclusively typical sequelae of certain pathological conditions involving a disturbance of mineral metabolism. The conditions that are significant in this connexion are similar to those described in the foregoing under the heading of rickets of atypical genesis. In analogy to what has been mentioned in that connexion, osteomalacia also appears in vitamin-D-resistant forms of varying and sometimes doubtful genesis.

sulphate in the cartilage cells and the adjoining part of the surrounding matrix in the resting cartilage. Over the proliferating zone, the zone of hypertrophic cells, and the zone of the provisional calcification, a marked diffuse labelling is seen, indicating uptake in both cells and cartilage matrix. Below the epiphyseal cartilage a zone of approximately equal size, which shows a relatively marked uptake, is seen. More detailed study of this zone shows uptake of radioactivity in the longitudinal bars of the cartilage shifted down to the metaphysis during the growth which has occurred after the injection of radiosulphate. Uptake of the radiosulphate is also observed in the regularly organized bone trabeculae below the epiphyseal cartilage (Plate IX*B* (*a*)). In the decalcified sections, the uptake of radioactivity was seen both in thin and in wide bone trabeculae, in the latter localized to their peripheral parts which were covered by osteoblasts. The latter areas probably represent the part of the matrix formed during the period radiosulphate was available in the body fluids. In some autoradiograms the zone of relatively high uptake below the epiphyseal plate contains a clearly-defined line parallel to the epiphyseal plate, which is more strongly labelled. This line corresponds to the part of the bone tissue labelled immediately after administration of the radio-sulphate, when the concentration of radiosulphate in the blood approaches its maximum. The line gradually shifts further down in the diaphysis as growth proceeds.

Autoradiograms of the growth zones of puppies affected by experimental rickets show the labelled zone corresponding to the epiphyseal cartilage to be wider than normal. This zone is, in some cases, more strongly labelled in the upper half, whereas the uptake in the lower half, corresponding to the hypertrophic cell zone, is considerably lower. The lower edge of the cartilage at the metaphyseal border contains a narrow transverse line which shows a marked uptake of radiosulphate (Plate IX*B* (*b*)). The zone below the epiphyseal cartilage, which in normal animals is shown to have a higher uptake than the bone tissue below it, in the rachitic animals, is as weakly labelled as the areas under it. Similarly, the line of high uptake, representing the structures labelled when the concentration of radiosulphate in the blood approached its maximum, is absent here. It appears, therefore, that no appreciable growth occurs in the rachitic animals in the interval between administration of sulphate and death.

The weaker labelling, apparent in some parts of the epiphyseal cartilage, may be due to a disturbance of chondroitin sulphate metabolism in the rachitic growth zones, but a closer specification of the mechanism of this deviation is not possible on the basis of our experiments.

D. X-ray Diffraction Studies

X-ray diffraction studies of normal and rachitic bone tissue have shown that the mineral salts consist of hydroxyapatite. The unit cells have the same dimensions as observed in normal bone tissue, *i.e.* a-axis 9·41 Å and c-axis 6·87 Å. The c-axis is oriented along the fibre axis of the collagen. In further studies, with micro-X-ray diffraction of osteoid tissue, it was demonstrated that this tissue in certain areas contains hydroxyapatite. Micro-X-ray diffraction of the osteoid tissue has, furthermore, shown an orientation of the crystallites perpendicular to the long axis of the bone (Engfeldt and Zetterström, 1955) (Plate X*A*). These observations may be correlated with the findings of Clark and Mrgudich (1934), who observed that the inorganic crystallites in rickets have lost their normal orientation parallel to the long axis of the bone.

E. Rickets During Healing

As mentioned in the preceding account, several studies have been devoted to rachitic changes as they appear during healing. In our investigations, the pattern of mineralization in cartilage and bone tissue has received particular attention.

Microradiographic studies were made of the border between cartilage and bone, in metatarsal bones and ribs from puppies affected by experimental rickets. The experimental animals were given 40,000 I.U. of calciferol daily for a period of 7 days. In this material, mineral salts are often seen in the longitudinal cartilage trabeculae at the border between bone and cartilage. Where such mineral deposit has occurred, it is evident that the capillaries, in their invasion of the cartilage, are guided along the mineralized walls of cartilage matrix. In some specimens, mineralization is restricted to the cartilage matrix at the border between cartilage and bone. In other microradiograms, deposition of mineral salts can also be seen between younger hypertrophic cells. In some microradiograms, mineralization of the cartilage between these areas is seen to be practically non-existent. In other specimens from the growth zone, the longitudinal cartilage trabeculae have been mineralized in the entire area between the young hypertrophic cells and the cartilage–bone junction. Occasionally, several parallel zones could be seen at short intervals from each other and perpendicular to the long axis of the bone, where alternating larger and smaller amounts of calcium salt have been deposited, possibly corresponding to the daily intake of calciferol.

Summing up, it can be stated that the mineralization pattern in the healing rachitic growth zone appears to present large variations. Mineralization of the cartilage matrix appears to be a prerequisite for normal

Microradiographic examination of ground sections from the defective teeth of these rachitic animals revealed that the areas where growth is most intense, as, for instance, the cervical parts of the carnassial and the permanent canines, contain large quantities of interglobular dentine which, so far as could be registered with the technique used, did not show any roentgen absorption. The globuli, although of different size, all display approximately the same roentgen density. With respect to mineralization, these globuli do not appear different from the dentine formed before vitamin-D deficiency was induced. A further observation was that the dentine formed in the crown of the lower carnassial during the experimental period, although displaying a normal pattern of mineralization, has a lower content of mineral salts.

In similar microradiographic investigations using ultra-soft X-rays on undecalcified microtome sections of normal and rachitic tooth germs, the presence of interglobular dentine and globuli in the rachitic specimens could be observed (Plate X*B* (*b*)).

The microradiographic studies of teeth and tooth germs revealed, furthermore, that the enamel defects vary in relation to the stage of development the enamel has attained prior to the experiment. In some teeth, the enamel has an uneven surface but mineralization is normal. In others, however, a poorly mineralized superficial zone can be seen in combination with hypoplastic areas.

Autoradiographic examination was also made of ground sections of teeth from both normal puppies and puppies with experimental rickets, which were given a single injection of radiocalcium or radiosulphate. In these investigations it was observed that the uptake of radiocalcium in the teeth of normal animals is localized to the dentine–predentine border, whereas the rest of the dentine is not labelled. In the normal tooth germs, two different patterns can be distinguished in the labelling. In addition to uptake of radiocalcium at the dentine border radiocalcium uptake is also seen as a narrow zone in the superficial enamel, or, in certain stages of development of the teeth, the enamel is labelled in its entire width. In the rachitic animals, the pattern of uptake of radiocalcium in teeth and tooth germs does not differ from that in the normal dogs. The labelled zone in the dentine–predentine border, on the other hand, is wider, due to the fact that radiocalcium is also taken up in the adjacent parts of the defective dentine formed during vitamin-D deficiency.

Autoradiographic studies with radiosulphate of ground sections of normal teeth and tooth germs showed that uptake occurs predominantly in the predentine and the pulp tissue, whereas a diffuse labelling can also be seen in a narrow zone at the dentine–enamel border. A slight uptake of radiosulphate can furthermore be observed in the enamel in the same area where radiocalcium uptake is found. The pattern of uptake of radiosulphate is essentially alike, in both the animals with experimental rickets and their normal controls. In the former, however, a slight uptake of the isotope is seen in the poorly-mineralized dentine of the crown formed during vitamin-D deficiency. In similar studies of decalcified sections indications of a disturbed pattern of uptake of radiosulphate in the predentine, and dentine could be observed in the rachitic tooth germs in healing state (Plate XI*A*).

Microradiographic examination of decalcified sections from the teeth of the normal and rachitic animals demonstrates a somewhat lower dry weight per unit area for the predentine zone as compared with the matrix in the mineralized dentine (Plates XI*B*, XII*A*). Particularly interesting is that a lower dry weight is also found for the dentine matrix between the globuli (Plate XII*A*). In addition, microradiographic examination of decalcified sections showed the mineralized dentine matrix, formed in the crown of the tooth germs during the period of healing, to have a somewhat lower dry weight than that formed earlier (Plate XII*B*). Study of the distribution of mineral salts in this part of the tooth germ revealed that the mineralization pattern is normal; no interglobular dentine is seen in this region.

VII. CONCLUDING REMARKS

From the investigations discussed in the foregoing, it appears that vitamin-D deficiency, in growing subjects, causes a disturbance of the mineralization of the hard tissues. Deposition of mineral salts in the matrix is arrested, but in many instances it does not appear to have ceased completely. As a result, broad osteoid seams which obliterate the bone trabeculae develop. Biophysical studies of osteoid in rickets have shown that its structure differs from that of the matrix in normal bone tissue. Accordingly, the arrangement of the collagen bundles is atypical as compared to normal bone tissue; in the Haversian systems, for instance, they appear in rickets to be partly oriented perpendicular to the Haversian canal. This conclusion is supported by roentgen crystallographic data indicating that the bone salt crystallites in the very poorly mineralized osteoid are oriented with their long axis at right angles to the long axis of the bone, in contrast to the crystallites in normal bone, which lie predominantly parallel to the long axis of the bone.

VI. DENTAL CHANGES IN VITAMIN-D DEFICIENCY

A. Introduction

Glisson long ago stated that rickets is associated with retarded dentition and pathological changes in the teeth, an observation which has later been confirmed by numerous authors. The rachitic changes in dental tissues have been studied, both on human material, and on experimental animals such as puppies, rats, and guinea-pigs, in which experimental rickets had been induced. It has been demonstrated that, in dogs and rats, the same conditions that are required for skeletal changes to appear, must be fulfilled before dental changes manifest themselves. Accordingly, in dogs vitamin-D deficiency suffices to produce rachitic changes in the teeth (Mellanby, 1929). In rats, it is induced, similarly to skeletal changes, by vitamin-D deficiency in combination with a low calcium or phosphorus content in the diet (Becks and Ryder, 1931; Karshan, 1933; Karshan and Rosebury, 1933). It would seem, however, that the bone tissue in these animals is more vulnerable than the dental tissue, since a high-calcium, low-phosphorus diet may cause defects in calcification in the former, whereas the teeth, on the whole, do not show any alterations (Karshan and Rosebury, 1933).

B. Histological Studies

The histological aspect of the changes caused by experimental rickets in rats have been described, among others by Becks and Ryder (1931), Rosebury and Karshan (1931), Kronfeld and Barker (1932), Karshan and Rosebury (1933), Euler and Kollath (1942), Boyle and Wesson (1943), Irving (1944). With respect to guinea-pigs these changes have been discussed by Howe *et al.* (1940), and, as they appear in dogs, by Mellanby (1929). The appearance of the teeth in human rickets has been histologically studied by, among others, Fleichmann (1910), Wolfe (1935), Wilton (1937), and Jump (1939). The changes are essentially similar in character, and consist of disturbances in the mineralization of enamel and dentine.

The first part of the tooth to show the effect of vitamin-D deficiency is the dentine. Under normal conditions, the odontoblasts initially form an organic, uncalcified tissue, known as the predentine. This formative process advances chiefly in one direction, towards the apical parts of the tooth. Because of this, the odontoblasts gradually withdraw towards the pulp as new layers of predentine are formed. In the organic matrix, subsequently, calcium salts are deposited in the form of small globuli which coalesce to form an, on the whole, uniformly mineralized dentine. Mineralization occurs consequently in the matrix, but not in the odontoblastic processes in the dentine. As the odontoblasts meanwhile form new layers of matrix, which take some time to become mineralized, dentine and odontoblasts are separated by a zone of unmineralized dentine, so called predentine.

In vitamin-D deficiency states the predentine zone is wider than usual, and irregularly delimitated from the dentine. In addition, the calcified dentine is seen higher up from the apex than under normal conditions (Fleichmann, 1910). There is, furthermore, less mineralized dentine than normal, and the total width of predentine and dentine is less than in healthy teeth. Wilton (1937) considered these changes due to abnormally slow differentiation of the odontoblasts. In addition to the widening of the predentine zone, the mineralization processes in the dentine zone deviate from the normal. The mineral salt globuli fail to coalesce on a normal scale, with the result that smaller or larger amounts of so-called interglobular dentine develop. This produces a pattern in which balls of mineralized dentine alternate with unmineralized interglobular dentine. The effects of vitamin-D deficiency on the enamel become manifest at a later stage than the changes in the dentine. In some areas of the enamel, mineralization becomes low or is entirely arrested; large defects may develop.

After administration of vitamin D, as mentioned in the foregoing, skeletal defects in the rachitic organism heal rapidly, and the structure is restored to normal conditions owing to constant regenerative processes. In teeth, on the other hand, the damage which has developed during the period of vitamin-D deficiency is permanent, since enamel and dentine are not rebuilt (Westin, 1926*a*, *b*; Wolfe, 1935). On histological examination, it is seen that the odontoblasts form new dentine, which is mineralized in the normal manner. This dentine is deposited between the interglobular dentine and the pulp. In this way, a more or less wide band of poorly mineralized dentine develops between the zones of dentine formed before and after vitamin-D deficiency.

C. Biophysical Studies

The biophysical techniques discussed, in the foregoing, with respect to their application in the study of bone tissue, have similarly been used in studies of the hard tissues of the teeth. These investigations have contributed several new observations on both normal structures and pathological changes. Such studies have been concerned, for instance, with the appearance of the dental tissue in experimental rickets in dogs (Engfeldt and Hammarlund-Essler, 1956).

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Similar informations concerning the structure of the so-called physiologic osteoid are unfortunately lacking. It is conceivable that the characteristics of osteoid in rickets merely reflect impeded development of the tissue (*cf.* Wilton, 1937). Normally, bone tissue at an early stage in its development forms longitudinally-arranged bundles of collagen, and *mineral salts* oriented along the fibre axis are deposited.

The morphological changes caused by rickets in the cartilaginous proliferation zone have been recognized for a long time. The irregular arrangement of the trabeculae is considered as a consequence of changes in the normal vascular invasion. The changed pattern of vascular invasion in rickets is probably related to the defective or irregular mineralization of the provisional calcification zone, calcification of the longitudinal cartilaginous trabeculae being essential to the normal process of vascular invasion in the cell columns. In this connexion it is also worthy of mention that the longitudinal growth of bone is considered to be impeded or completely arrested (Wilton, 1937; Dodds and Cameron, 1934, 1943).

Studies, made in our laboratory, of dentine mineralization have demonstrated that the predentine zone has a somewhat lower dry weight per unit area than matrix in the mineralized dentine. The same applies to matrix in the unmineralized parts of the dentine seen in vitamin-D deficiency. The significance of this observation is somewhat doubtful. A possible explanation could be that, during the mineralization process, organic matter is added to the matrix simultaneously with deposition of mineral salts.

Morphological and metabolic data show that the main principles in the mode of action of vitamin D are established. Accordingly, it seems to be generally agreed that vitamin D influences the intestinal resorption of calcium (Carlsson, 1952; Nicolaysen and Eeg-Larsen, 1953, Carlsson and Lindqvist, 1955; Bauer *et al.*, 1955, Snapper, 1957). With respect to the effect of the vitamin on bone tissue opinions, generally, coincide, even though certain details may be subject to controversy. Lindqvist (1959) considers vitamin D and parathyroid hormone the primary factors responsible for maintaining normal calcium and phosphorus values in tissue fluid and blood. This effect of vitamin D is considered due to the ability of the vitamin to stimulate bone resorption (Carlsson and Lindqvist, 1955; Bauer *et al.*, 1955; Lindqvist, 1959). Nicolaysen and Jansen (1939) and Mellanby (1949) also consider vitamin D to have a specific effect on bone structure. It has furthermore been claimed that vitamin D influences kidney maintenance of calcium and phosphorus (Harrison and Harrison, 1941; Snapper, 1957). The accretion of bone salt, apparent in rachitic animals after administration of vitamin D, appears, at least in part, to be secondary to the increased $Ca \times P$ product in the body fluid (Harris, 1956; Snapper, 1957).

The available morphological data do not permit any conclusions concerning the effect of vitamin D on the cellular or biochemical level. With respect to the biochemical aspect of vitamin-D activity, it has been shown that phosphorylated vitamin D_1, when added to a suspension of kidney mitochondria, increases oxygen consumption (Zetterström, 1951). This phenomenon is thought due to activation of phosphatase. In recent years it has been established that vitamin D administration increases the citrate content of urine and certain tissues (Wassjö and Larsen, 1951; Carlsson and Hollunger, 1954). The observation that kidney mitochondria, from rats fed a vitamin-D-deficient diet, showed a reduction of the oxidation of citrate in the presence of a phosphate acceptor, may be of interest in this connexion (De Luca *et al.*, 1957). It is suggested that the citrate medium may be an important factor in the determination of the transport and deposition of calcium in the process of calcification.

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9

Fluorine

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Contents

I. HISTORICAL

Research workers have interested themselves in fluorine since the middle of the nineteenth century, and, from the very beginning of their acquaintance with the element, they have commented on its untoward effects. The first appearance of its toxicity was, in fact, due to hydrofluoric acid. However, Rabuteau (quoted by Gysel) reported, as early as 1867, that he had observed toxic effects of fluorides on animals. After some self-medication with 0·25 g of sodium fluoride, he noted slight giddiness, increased salivation, anxiety and pruritus lasting for several days. Schlusser (also quoted by Gysel) studied the effects of calcium fluoride from 1873 to 1898 and established, doubtless for the first time, that, in excess, it produced the same symptoms as those produced from its deficiency.

During the same period, fluorine began to be advocated for the prevention of caries, indeed, calcium fluoride made into tablets was used to ward off caries in England (1865) and Germany (1870). In 1896 there appeared a report on eight years' continuous trials of calcium fluoride, which showed positive results, but with no outstanding success, probably, since fluorine did not at that time become established as a prophylactic (Rost, 1955). After Roholm's (1939) publication on fluorine damage, all fluorine supplementation was forbidden by the health authorities in Germany; indeed, some "defluorization" was undertaken.

An interesting communication came from a medical man, Dr. Alfred Deninger (1896), who gave his wife, during her first pregnancy, finely-powdered calcium fluoride (in amounts that would go on the "tip of a knife") in her soup. He wished to protect the young mother's dentition from erosion, and to make available, to the growing infant, adequate reserves for tooth production. Since then, four generations of Deningers have been treated with small doses of calcium fluoride; all have been characterized by excellent dentition (Mrs (Dr) Stresemann-Deninger, private communication).

The early part of the twentieth century saw the appearance of many reports about the reduced tendency to caries of teeth with pitted enamel (Black, 1916; McKay, 1929; Dean, 1938; and many others). accompanied by the recommendation to administer fluorine for long periods as a protective agent against caries.

II. THE TRACE ELEMENTS

1. Are They Essential?

Up to the beginning of this century more or less simple explanations were given of the biochemical processes occurring in human and animal species, also in plants. Biochemical thinking was macrochemical. It was held that living matter contained three important types of substance—carbohydrate, fat and protein. Only simple methods of investigation were as yet available, and the behaviour of substances of these three types could be studied calorimetrically in human and animal organisms. However, it was known already that water was essential to chemical reactions, but its importance in metabolism, unlike that of the other substances mentioned, was not easily established. That the water was not simply a source of hydrogen and oxygen, but also carried with it various constituents, was not envisaged.

At the beginning of this century it was Hopkins who, in 1906, made his prophetic announcement that certain other substances, though present in minute quantities, would be found also to participate in nutrition. He thus prepared the way for the subsequently discovered vitamins.

It is only in recent years that we have become aware that another large group of substances, also present in extremely small quantities, are likewise essential to the normal development and health of the body. This group consists of numerous elements present in the atmosphere, the soil, water, and foods at such low concentrations that we refer to them as "trace elements." The significance of only some of these elements has so far been elucidated. Investigation of the whole group is, however, at present in a state of flux, proceeding parallel with the development of micro methods. Thus, to take only one example, iodine metabolism has been understood only since it has become possible to determine iodine in quantities of 0·1 μg or less.

Trace elements are ubiquitous. Certain ones, we know, are constituents of enzymes or hormones, and therefore indispensable to the organism. The role of these particular trace elements has been clarified. However, there are to be found, in vegetable as well as in human and animal organisms, all the elements of the periodic table, though it is not yet known whether or not they are all of biochemical significance.

has been shown to be false. There is, however, some support for the view that a constant relation persists between the amounts of bromine and chlorine in the body (Damiens and Blaignan, 1920), but the position has still to be clarified.

Fluorine also is a regular constituent of the inanimate and animate worlds. It is found in water, in soil, and in the living organism, more particularly in bones and teeth. However, several generations of rats can be raised on a diet entirely free of fluorine without the least adverse effect of any vital function. As we shall discuss below, anticariogenic activity has been attributed to fluorine. In contradistinction, however, to what is observed in the truly "essential" elements, it is never, even according to the most optimistic accounts, 100 per cent effective. Most authors credit fluorine with a 30 to 40 per cent success; indeed this value has almost become a "magic number," since all investigators attempt to evaluate their results statistically in comparison with it. It cannot so far be stated, with confidence, that fluorine is an element essential to life.

2. The Natural Distribution of Fluorine

Fluorine is widespread throughout the world. It is to be found in several minerals of the earth's crust, as fluorspar (fluorite, CaF_2), as apatite ($3Ca_3(PO_4)_2$, $Ca(F, Cl)OH)_2$, also as cryolite (Na_3AlF_6). Other minerals also contain fluorine. It is found in the soil. Von Fellenberg (1948) reported fluorine in amounts ranging from 9·8 to 29·6 mg/100 g in six different samples of Swiss soil. The higher the pH, the higher the fluorine content, from which it may be inferred that alkaline soils are the more effective in retaining fluorine. As much as 60 mg/100 g has been found in North African soil (Charnot and Langlais, 1939). The fluorine-rich dust from this soil is the cause of the disease "darmous," widespread in North Africa.

Obviously, water from fluorine-containing soil will itself contain fluorine, and in quantities that will vary from place to place. In the United States no less than 335 localities, in 25 different states, have been found to have "drinking-water fluorosis," that is, there is more than 1 p.p.m. fluorine in the water. Fluorine is also present in various plants. Figures for the fluorine contents of plants are not always consistent, either because unsuitable methods of investigation have been used or, as von Fellenberg (1948) holds, because *calcium hydroxide, incompletely freed of fluorine,* has been used for ashing. Indeed, he himself found 19·2 μg of fluorine/g in a calcium hydroxide of analytical reagent quality. We shall not quote the results of older investigations, but will give a few examples from the figures reported by Mayerhofer, Schneider and Wasitzky (1932). The values must not be taken as fixed, for the plant is under no necessity to contain constant amounts of fluorine. The quantities present depend on locality, on the fluorine content of the water, and on various other factors. Only a small selection of the values is given in Table 9.3.

TABLE 9.3
Fluorine in Foods, etc.
(mg/100 g)

Material	Fresh weight	Dry weight
Spinach	0·021	0·169
Lettuce	0·010	0·125
Onions	0·048	0·304
Runner beans	0·006	0·058
Cucumber	traces	
Tea		0·475
Coffee		0·250

Almost 70 per cent of the fluorine in tea is water-soluble; in coffee, the amount may reach 80 per cent.

Reid (1936) found in Chinese foods, fluorine levels, expressed as mg/100 g dry weight, of 0·07 to 0·09 in rice, 0·17 in buckwheat, and 0·40 to 0·67 in soya bean. Attention is called to other values found in various plants from Virginia (Robinson and Edginton, 1946).

Similarly, fluorine has been found at various high and low concentrations in nearly all foods and feeding stuffs. As already mentioned, one must not expect constant figures from these determinations; in the same way, the water from a well or stream will not always show the same fluorine content, which may indeed vary from hour to hour. Further, it is not yet known in what form fluorine exists as a constituent of, for example, plants. It is generally stated that fluorine in plants is present partly in an insoluble and partly in a water-soluble form. One can consequently say nothing about the toxicity of these forms of fluorine; it is assumed that they have no specific toxicity. In one plant, however, *Dichapetalum cymosum,* which has caused serious losses of stock animals in South Africa, the presence of monofluoroacetic acid, $CH_2F.COOH$, has been established. At present no biological significance is attributed to fluorine in plants: most authors deny to it any growth-stimulating properties.

We repeat again, that one must treat the results of quantitative fluorine analyses, especially those reported in the older literature, with some scepticism. For quantitative investigations carried out more recently we accept the view of Hammond and McIntire (1940) that "quantitative determination of fluorine has been recognized as a difficult and tedious procedure."

Lang gives, in a 1952 publication, the list of trace elements shown in Table 9.1. This survey still has

TABLE 9.1

Physiological function established	Physiological function unsettled	Physiological function absent	Toxic
Iodine	Aluminium	Boron	Lead
Cobalt	Arsenic	Bromine	Mercury
Copper	Chromium	Caesium	
Manganese	Gold	Fluorine	
Silicon	Molybdenum	Lithium	
Vanadium*	Nickel	Rubidium	
Zinc	Silver	Strontium	
	Titanium		
	Tin		

* Needed only by lower animals.

validity, although it might be added, it would perhaps be better to head the third group "Physiological function not yet established" rather than "Physiological function absent." It is in this group that we find fluorine, with which we are here particularly concerned. This is indeed the whole problem of the trace elements: all of them are found in the animal organism, but we cannot as yet specify their significance.

From the same publication by Lang (1952) we may take figures for the daily balance or turnover, though we must not endow them with mathematical precision. Many of these trace elements appear in the organism as the result of industrialization; larger or smaller quantities of them are found according to the circumstances. However, Table 9.2 does give an approximate picture of the amounts of the trace elements entering and leaving the organism.

TABLE 9.2

Element	Dietary intake (mg/day)	Daily excretion (mg)	
		Urine	Faeces
Aluminium	10 to 40	0·04 to 0·1	10 to 40
Arsenic	0 to 1	0·00 to 0·2	—
Lead	0·29	0·01 to 0·02	0·3
Boron	9 to 20	9 to 20	—
Bromine	—	3 to 5	—
Fluorine	0·3 to 1·5	0·3 to 1·5	—
Cobalt	—	0·03	0·15
Copper	2 to 3	0·02 to 0·03	2 to 3
Lithium	1·6 to 2·3	0·7	0·7 to 2
Manganese	3 to 4·5	0·01 to 0·02	3·5 to 4·5
Nickel	0·25 to 0·42	0·14 to 0·25	0·1 to 0·2
Silver	0·088	0·00	0·058
Zinc	6 to 40	0·03	3 to 20
Tin	10 to 20	0·01 to 0·02	20

Whether certain of the trace elements are essential for life cannot in the light of present-day knowledge be given a complete answer. To answer it for any given element we must know whether or not a deficiency disease results from its absence. This was found to occur with cobalt. Cattle and sheep, in both Denmark and Australia, developed a characteristic disease, described in the literature as "Denmark wasting disease" or "enzootic marasmus," which is unequivocally cured with cobalt. Today we understand its pathogenesis and, knowing that cobalt is an important constituent of vitamin B_{12}, we can say with certainty that cobalt is essential for haemopoiesis in the human or other animal organism.

Copper is equally essential to life, being indispensable to haemoglobin formation. It is always to be found in the blood at a level of 100 to 150 μg per 100 ml. Among the enzymes, polyphenoloxidase contains copper and certain dehydrogenases are inhibited by copper.

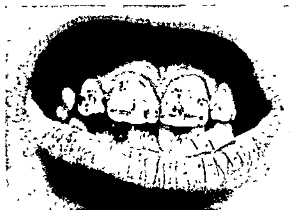
Zinc is a constituent of certain enzymes. The phosphatases and carbonic anhydrase both contain zinc, so does insulin, and zinc also plays an important part in the treatment of diabetes with protamine-zinc-insulin. Similar remarks apply to manganese, since animals deprived of this element exhibit reproductive disturbances.

Thus, the significance of the elements in Column 1 of Table 9.1 has been established both experimentally and clinically.

When, today, we re-examine the list of elements in Column 3 of Table 9.1, we find it necessary to remove boron, which we now know to be essential for plant growth. Because it is necessary for the plant, man and other animals may be said to profit indirectly from its presence, though it also occurs in the animal organism. On diets poor in boron, rats show no deficiency symptoms, and small additions of boron (155 μg/kg) bring about no improvement in the rat's physiological condition (Teresi *et al.*, 1944). Thus, boron is probably dispensable to man and animals.

Bromine is evidently needed by lower animals. The purple snail produces dibromo-indigo. Anthozoa elaborate bromogorgonic acid (dibromotyrosine). Bromine occurs regularly in human and other animal organisms. Bernhard and Ucko (1926) found large amounts of bromine in the hypophysis (of the dog), even as much as 12·5 mg, or more, per 100 g. The view that the organism gets rid of bromine during sleep and that it is in some way connected with sleep

(a) Mottled Teeth. Girl from Campagnano di Roma
F amount in the water 3–4, 4 mg/l.
(*Wespi*, 1954)





(b) Darmous

(c) Autoradiogram of Chromatograms of Diiodotyrosine made Radioactive by Exchange with ^{131}I Treated with Equimolar Quantities of Different Halogene Ions
(*Minder and Gordonoff*, 1956)

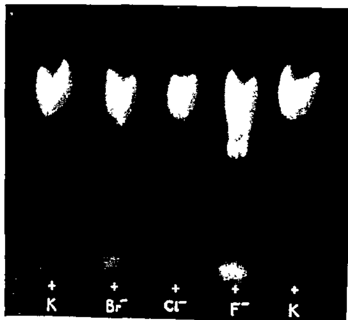


Plate XIII

Unfortunately, there are to be found in the literature figures presented without any indication of the methods used to get them, so that valid comparisons of different authors' figures may be impossible.

We would end this section with the remark that fluorine is a ubiquitous element, and that we have all the same no way of assessing its physiological significance.

Certainly one of the best evaluations of fluorine is that of von Fellenberg (1951). A survey of the different analytical methods has been given by Epars (1954).

III. FLUORINE AND ENZYMES

It is well known that the fluorine ions are able to inhibit enzyme processes. Physiologists and pharmacologists have a preference, however, for fluoroacetate. The toxicity of fluoroacetic acid is not dependent on the fluorine atom; the whole molecule is toxic. Indeed, the toxicity of fluoroacetic acids is strongly reminiscent of that due to mono-iodoacetic acid.

We shall, without any attempt at completeness, refer to certain enzymes that are inhibited by the fluorine ion as such. When this occurs the fluorine ion may be required only in traces. It has also been demonstrated that fatty acid oxidase activity is inhibited by 0·01 mol fluoride (Johnson and Lardy, 1950). The closely related acetate activating system is inhibited *in vitro* by 0 0001 to 0 0005 mol fluoride (Aisenberg and Potter, 1955). The same concentration of fluoride is present in soft tissues under conditions of fluorosis. Lipases are inhibited by fluorine (Rothschild, 1929); the inhibition is stronger the more acid the solution. Lecithase is insensitive to sodium fluoride (Ogawa, 1936). Fluorides inhibit the tyrosine-tyrosinase and the dopa-tyrosinase reactions (Lerner, 1952). Succinic dehydrogenase is inhibited by fluoride in the presence of inorganic phosphate (Slater and Bonner, 1951).

Fluorine interferes with the function of the gastric mucous membrane. Sodium fluoride abolishes the effect of histamine on acid production. When the sodium fluoride is removed, the histamine effect returns (Bowie, Darlow and Murray, 1953). Of the succinic dehydrogenases, fluorine inhibits fumaric dehydrogenase, also enolase (Jakobson and Tapadinhas, 1935).

Respiration is affected only by large doses of fluorine. In chickens, doses that can be fatal produce hyperpnoea; in other animals an increase in the frequency and amplitude of respiration, after high doses, is succeeded by their retardation and, finally, respiratory paralysis.

The activity of cholinesterase is affected only by large doses of fluorine. On a diet containing 0·05 per cent sodium fluoride there was no effect on the amount of cholinesterase in the brain (Dybing and Loe, 1956). The fluorine ion has been shown experimentally to inhibit the incorporation of phosphorus in cat brain-slices (Strickland, 1954). In muscle metabolism there is inhibition of lactic acid formation. Presumably, according to De Eds (1933), a labile precursor of lactic acid (hexosemonophosphate) is converted into a stable compound (glucose-6-phosphate).

Blood coagulation is inhibited by the fluorine ion, 0·15 per cent of sodium fluoride being already sufficient to stop it entirely. This property of fluorine was naturally attributed to its ability to precipitate calcium, in accordance with the coagulation scheme of A. Schmidt. However, it is not a matter of calcium precipitation, but of a specific activity whereby the production of fibrin is hindered (Roholm, 1936). According to Lumière and Sonnery (1934), 0·3 g of sodium fluoride render 100 ml of blood uncoagulable. When blood is mixed with sodium fluoride, normal haemolysis is inhibited (Iljin, 1936). Fluorine inhibits the conversion of added glucose into galactose and lactose in slices of actively-secreting mammary gland (Grant, 1936).

Interest has also been shown in the effect of fluorine on salivation. In poisoning with fluorides an increase in salivary secretion is observed (Muehlberger, 1930). Salivary amylase is said not to be affected (McClure, 1939), but the fluorine ion interferes with retinal amylase at concentrations of 1:1,000 (Trematori, 1936). In general, the fluorine ion inhibits the enzymes involved in carbohydrate catabolism (Bramstedt, Krönke and Naujoks, 1956). It was thought possible to connect this inhibitory activity with the effect of fluorine on caries. The authors last quoted invalidate such a view, if only on purely quantitative grounds, since enzymic inhibition is first to be seen only at a concentration of 190 mg/litre. On the other hand, they were able to demonstrate that fluorine concentrations of 1 to 2 mg/l significantly raised aerobic and anaerobic glucose degradation in caries. In their view, the retarded carbohydrate turnover in patients suffering from caries is accelerated in the saliva.

The acid phosphatases are inhibited by fluoride. Lammers and Haffer (1956) find a pH optimum of 5·5 for this inhibition, though concentrations found in drinking water can be inhibitory. Phillips (1932) was able to demonstrate an increase of phosphatase in the

blood, but a reduction in the bones. In general, phosphatases are sensitive to fluoride. Since the phosphatases play a part in the calcification processes, it was thought possible to explain, in this way, the bone disturbances occurring in fluorine poisoning. Opposed to this view is that of von Euler and Eichler (1942), who found similar disturbances of the bones from a fluorine compound without enzymic activity (fluorotyrosine). The fluorine ion itself affects only the acid phosphatases, but there are said to be fluorine-containing compounds that also affect the alkaline phosphatases of the bone, without causing any disturbances in calcification. With a view to the topical use of fluorine, Naujoks (1957) investigated histochemically the effect of alkaline and acid phosphatases on human gums before and after treatment with fluorine: the alkaline phosphatases were partly inhibited, the acid phosphatases not at all.

As a protoplasmic poison, the fluorine ion possesses also bactericidal properties. Sodium fluorine inhibits *Torula cerevisiae*, *Bacterium prodigiosus* and *B. typhi* as well as pneumococci and streptococci. Higher concentrations, however, are involved, 1:100, 1:150 and1:300 (Hewelke, 1890). There have clearly been attempts to explain the anticariogenic action of fluorine in terms of bactericidal action. The relationships of *B. acidophilus* have been thoroughly studied. Concentrations of sodium fluoride of 10^{-4} inhibit acid production in culture, but a reaction in acidophilus has also been seen after teeth have been brushed twice (Shaner and Smith, 1946). Some authors have observed no effect from the action of fluorides over a short period, others only after prolonged additions to conductivity water, but these results also have not been confirmed, so that one can only say, broadly, that the anticariogenic effect of fluorine ions can hardly be explained by its effect on the oral flora and in particular on *B. acidophilus* (Knutson, 1947).

In general, fluorine ions depress metabolism. On this depends the observed activity of fluorotyrosine on hyperthyroidism, and it also explains the observed late dentition after the action of fluorine in quantities above 2 mg of sodium fluoride/litre. The action in depressing metabolism can also be observed on the tadpoles of the South African clawed toad (*Xenopus laevis*). If concentrations of 1 mg/l are present in the water, the tadpoles become broader and longer than the controls, and metamorphosis is also delayed.

Braun (1952) observed a clear thyreostatic effect of a glutin-fluorine complex on tadpoles, with postponement of growth and metamorphosis.

Unfortunately, no study of the effect of fluorine on caries in secondary dentition has been undertaken. All statisticians have concerned themselves with the decline of carious processes, but nothing has been forthcoming on this fundamental question of *dentitio tarda*.

In summary, we may say that fluorine acts as a protoplasmic poison, and sometimes has an effect on enzymatic processes, blocking carbohydrate catabolism, among other cycles. It is reasonable to assume that the fluorine ion can affect, in some way also, the citric acid cycle, though the exact conditions, particularly in connexion with the anticariogenic properties of fluorine, have still not been unequivocally explained. Experiments carried out with fluoroacetate do not, in this field, apply also to the salts of fluorine.

IV. PHARMACOLOGY OF FLUORINE

O. Schmiedeberg, who founded experimental pharmacology, defines pharmacology as the study of those changes brought about in the living animal body by chemically active substances. Pharmacology follows chemical compounds through the human, and animal organism, and establishes the changes occurring in the individual organs as well as in the organism as a whole. If one attempts to inform oneself about such changes in fluorine compounds by consulting the text books of pharmacology and, in the first instance, those written in Europe, one comes up against greater or lesser difficulties. Either fluorine is not mentioned at all, or attention is directed to its toxic properties only. Thus, Møller (1958) writes in his text book (p. 718), just published, that fluorine is strongly toxic, that it precipitates calcium and inhibits enzymatic processes, particularly the activity of the phosphatases. It acts locally in disturbing tissue function, and this is not a question of acidity but is true for undissociated fluorides. The mechanism of its action in caries is unknown. This most important problem has been in no way settled. Consequently it is difficult to draw practical conclusions. "It is necessary everywhere most seriously to consider the proposal to add fluorine to drinking water containing only a small amount of this element, for such a measure might involve serious dangers for the population as a whole" (Møller).

After examining the experimental investigations, it must be allowed that the toxic actions of fluorine cannot be gainsaid. Moreover, caries is generally accepted as a "disease of civilization," which is certainly connected with faulty nutrition and, in particular, with misuse of sugar. Fluorine can only be an adjuvant in the treatment of caries, which is caused by a multitude of conditions and is in no way

be applied to fluorine metabolism. All investigators agree that fluorine is an element taken up rapidly, and slowly eliminated. An accumulation of fluorine occurs, and it is impossible, or nearly so, to establish this by means of a short-lived element, such as ^{18}F, *having a* half-life of 112 min. In doing so, one risks accepting results that have only poor validity for fluorine metabolism in general.

Fluorine acts also on the vascular system, but only in high doses. Richardson, Muhler and Bishop (1955) produced a reduction in the dog's blood pressure with 5 mg sodium fluoride by mouth, but found no effect with 1 mg, the kind of quantity to be found in fluoridized water. Parallel with a reduction in blood pressure are certain other changes in heart rate, pulse quality and rate of blood flow, as one finds in general with drugs that reduce the blood pressure. Tin fluoride, in addition, reduces rectal temperature.

Fluorine passes through the placenta into the embryo. After the addition of fluorine, higher values are found in the placenta than in the umbilical blood stream. Gardner, Smith, Hodge, Overton and Feltman (1952) hold the view that the placenta acts as a barrier, concentrating fluorine, because this could be toxic to the embryo. Held (1952) also found the placenta to be permeable, for the fluorine picture in the umbilical vein corresponds with that of the maternal blood. No correlation was found between the fluorine contents of the milk and the maternal blood. Feltman and Kosel (1955) attribute to the placenta the ability to store fluorine. In any event, foetal blood becomes richer in fluorine as the values for the maternal blood increase. When fluorine is administered during pregnancy, the fluorine level in the maternal blood rises, as also in the milk, though the increase in the latter is less than in the former.

Fluorine is eliminated in the urine in amounts more or less parallel with those taken up. According to Smith, Gardner and Hodge (1950), the fluorine content of the urine increases from 0·06 to 0·12 p.p.m. when the amount in the water increases from 0·16 to 0·36 p.p.m. In experiments on rabbits, Gardner, Scharff, Smith and Hodge (1957) established that half the fluorine administered remains behind, and that of this half probably 76·5 per cent is locked up in the skeleton.

According to Likins, McClure and Steere (1956), the elimination of administered fluorine in man is more extensive if large amounts of fluorine have previously been received. From this there derives a re-elimination of stored fluorine, and it thus seems likely that not all the fluorine built into the bones is there bound irreversibly; rather, there takes place an exchange with hydroxyl ions, more easily so in young than in old individuals. In Table 9.4 (p. 221), taken from McClure (1939*a*), are represented the conditions

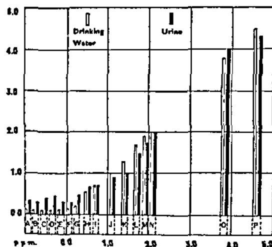


FIG. 9.1. RELATION OF FLUORINE IN DRINKING WATER TO FLUORINE CONTENT OF URINE (*McClure* 1948)

in the urine; with large amounts, some fluorine remains unexcreted (Fig. 9.1). Smith, Gardner and Hodge (1955) established changes in excretions of rabbits in which an acute nephritis had been produced with uranyl nitrate.

Pindborg (1957) fed rats on a diet containing 0·05 per cent sodium fluoride. In the kidneys there were changes in the form of dilatation of Henle's loops, a flattening of the epithelium in the damaged tubules, and a dilatation of the tubules, probably due to blockage in Henle's loops. These changes occur with more frequency in young than in old rats.

That fluorine is laid down in bones and teeth has been known for a long time. The first signs of increased deposition of fluorine in the structure of the tooth is "mottling." The symptom can be evoked by as little as 2 mg/l, but is also noticeable with smaller amounts. There was justification for the remark by De Eds (1932) "in view of the definite possibility of chronic fluoride intoxication, a note of warning is in order." (Driak (1952) found less fluorine in carious teeth than in healthy ones. The fluorine content of the enamel increases from incisors to molars, but, in the dentine, increases from molars to incisors. Driak maintains that teeth with a high fluorine content are not caries-resistant. In bones and dental tissue, fluorine increases with age (Schmidt, 1952). If opaque spots occur on the enamel, fluorine administration must be stopped. Elimination of fluorine in the urine continues for a long time. In cryolite workers, fluorine was found in the urine long after they had ceased working (Largent, 1952).

Shaw, Gupta and Myer (1956) found high fluorine contents in the teeth of Indians in Boston, either

to be attributed to a single factor. The trials of fluoridizing drinking water, which began in 1945, can only be properly surveyed by the time a new generation has grown up with the "benefit" of fluoride-containing drinking water and has shown that their teeth, between the ages of 20 and 40, have remained free of caries. Such information will not become available before the year 2000 (Kruse, 1954).

As already indicated, fluorine produces local damage. Oral administration causes alterations of the gastric epithelium, with haemorrhages in rabbits and cats. In the dog gastro-enteritis results, naturally only after large doses. If rabbits are treated with atropine, directly into the eye, and given 0·5 per cent sodium fluoride, there follow dilation of the capillaries and an earlier onset of mydriasis (La Fluoresta, 1940).

Tadpoles show a reduced development, to the extent of 10 per cent, in a solution containing 8–10 per cent of sodium fluoride. If they are then put into conductivity water, subsequent growth is markedly accelerated (ten Cate, 1940).

Tappeiner (1889) observed fibrillation of the frog's striated muscle after treatment with 0·01 g of sodium fluoride; he attributed this to excitation of the motor-nerve endings. After 0·02 g, paralysis of the central nervous system occurred. The muscles go into spasm, and the frog succumbs to heart failure. In the rabbit, he established a paralysis of the vasomotor centre after 0·5 g by mouth or 0·15 g parenterally, preceded by preliminary spasms of the vascular system.

Volker, Sognnaes and Bibby (1940) observed, in the frog, an increase in heart rate and an inhibition of systole. In warm-blooded animals 25 mg/kg of sodium fluoride produced cardiac dilatation, ventricular vibration and paralysis of the vascular system and, sometimes, local necroses in the myocardium were found.

Schulz (1936) concerned himself with the action of fluorine on the isolated heart and established damage consequent upon precipitation of calcium; oxalic acid acts similarly. Addition of calcium restored the heart to activity. Also, in isolated guinea-pig gut, there occur changes that are associated with the precipitation of calcium. In the mouse, a subcutaneous application of 0 04 mg/g causes muscular twitching, and 0 05 mg/g is the minimum lethal dose, which thus lies close to the minimal amount that has any action at all. A dose of 0·07 mg/g is always lethal; it elicits fibrillation in the muscles, and cramps, and it results in death a few hours later. The oral toxic dose is the same as the subcutaneous. Fluorine has also been applied to isolated organs. Thus, Ewig (1929) established that respiration of liver or kidney slices was not affected, although glycolytic fermentation was. Hintzsche (1953) produced an 18 per cent reduction in growth of ten-day-old chick embryos treated with 1 p.p.m. of sodium fluoride, the reduction with 0·25 p.p.m. being 24 per cent; these figures were statistically significant.

The fluorine content of individual organs was studied at an early stage. Tamman, in 1888, found fluorine in individual organs, but we shall not quote his figures, since methods at that time were not satisfactory. Even now, the determination of fluorine in organs leaves much to be desired (von Fellenberg). Of recent work I shall refer only to that of Suttie, Phillips and Miller Jr. (1958), who found 2–3 p.p.m. of fluorine in various organs—heart, liver, kidney, pancreas, thyroid and adrenals—after administration of 3–5 p.p.m.

Since the preparation of radioactive fluorine, ^{18}F, it has been possible with comparative ease and speed to detect fluorine in the organs, and to investigate fluorine metabolism. Volker, Sognnaes and Bibby (1941) injected fluorine intraperitoneally into rats and cats. Fluorine was detectable, 45 min later, in the skeleton and teeth, but only in the growing roots of the latter, and more markedly than in the jawbone or the femur. No fluorine could be detected in the tip of the incisors and none was observed in the crowns of the cats' teeth. Myers, Hamilton and Becks (1952) established by means of radioactive fluorine that the element accumulates only on the surface of defective teeth.

Perkinson Jr., Whitney, Monroe, Lotz and Comar (1955) injected lambs and a cow intravenously with radioactive fluorine. The fall-off in radioactive fluorine corresponds with that of radioactive calcium. Oral uptake results in rapid absorption, the maximum being after 3 hours, in lambs, and after 5 hours, in the cow. Localization in the bones was similar to that of radioactive calcium. In laying hens, fluorine is most abundantly present in the shell, and least abundantly in the yolk. Fluorine is also detectable in the milk.

Durbin (1954) made extensive measurements of fluorine contents after administration of radioactive fluorine. She found particularly high values for kidneys and bones. Complete uptake had not occurred after 9 hours; grown rats deposited less in their teeth and bones than did young ones, the accumulation in the bones depending on the degree of vascularization, and the rate of growth. Although Durbin maintains that the thyroid does not take up fluorine, her own tables contradict this. She established that, after 15 min, 0 001 per cent of fluorine had been taken up by the thyroid, 0 002 per cent after 1 hr, 0 001 per cent after 4 hr and 0·004 per cent after 9 hr, that is, that the small thyroid gland takes up increasing quantities with time.

One may legitimately raise the question whether the results obtained with radioactive fluorine should

TABLE 9.4

Category	Dose frequency	Amount	Time	Clinical effect
Acute poisoning	Single	5–10 g	2–4 hr	Death
Chronic high-grade poisoning	Daily	20–80 mg or more	10–20 yr	Crippling fluorosis
Chronic low-grade poisoning, mottled enamel	Daily	2–8 mg or more	Daily during first 8 yr	Mottled enamel
Preventive dentistry	Daily	1–1·5 p.p.m. in water	First 8 yr and later	Decreased dental caries

V. TOXICOLOGY OF FLUORINE

Fluorine is certainly the most toxic of the halogens. In the literature, there are references to the toxicity of fluorine compounds in general, but there are degrees of toxicity among them. We shall not here be considering all fluorine compounds, as, for example, fluoroacetate, fluorobenzene. In these compounds the whole molecule is toxic. We are, however, concerned with the toxicity of the fluoride ion, for it is only in this form that fluorine is recommended to combat caries; it is in this form also that fluorine occurs in water and minerals, and in this form that man and animals take it up.

As indicated in Section IV, "Pharmacology of Fluorine," the element should be regarded as causing a calcium shortage, which must lead to bone decalcification. Fluorine also affects enzyme activity: it inhibits carbohydrate metabolism because it blocks the liberation of hexose mono- and di-phosphoric acids, which depresses lactic acid formation. Cholinesterase is also inhibited, so that the organism produces an excess of acetylcholine, with increased vagotonicity. The toxicity of fluorine is not due solely to these actions on enzyme systems, but predominantly to the disturbances it causes in calcium metabolism. Any organism having increased calcium requirements, as in pregnancy or lactation, will react more sharply to a given amount of fluorine than will an organism in calcium balance. Man is more sensitive to fluorine than are animals in this matter of calcium requirements.

Our knowledge about the toxicity of fluorine is, in particular, due to Roholm (1939), who has collected all the available information in an admirable monograph. He was the first to call attention to the fact that a daily intake of as little as 0·1 mg fluorine per kg can bring about chronic intoxication. Pitted enamel can result from the presence of more than 1 mg/l in drinking water; indeed, cases of "mottled teeth" have been reported in individuals receiving less than that amount.

Fluorides can act topically, if the skin reaction is acid, producing abrasion. For this reason oral uptake may lead to gastric abrasions, and nausea. Subcutaneous administration of even neutral solutions can lead to necroses, inflammation and ulcers.

Roholm in classifying the forms of toxication distinguishes:

1. Local irritation of skin and mucosa. Dalla Volta (1924) observed *reddening and swelling of the skin* after subcutaneous injection of a 2 per cent sodium fluoride solution. Repeated injections led to hardening of the skin and abscesses, with fissures.
2. Acute toxication by absorption after oral uptake, especially of the more soluble fluorine compounds.
3. Chronic toxication from absorption leading to—
   (*a*) degenerative dental changes.
   (*b*) generalized osteosclerosis.
   (*c*) osteoporosis, accompanied by cachexia.

Roholm distinguishes between two forms of chronic toxication in man—

(*a*) Pitted enamel: an enemic hypoplasia of permanent teeth, occurring all over the world, and generally evoked by a high fluorine content of the water. The damaging factor acts during tooth formation, that is, in childhood.

(*b*) Generalized osteosclerosis in adults, *resulting* from year-long regular consumption of fluorine in large amounts. Signs may occur simultaneously in both bones and teeth.

1. Mottled Enamel (Plate XIII (*a*))

Mottling of the teeth is the most widespread symptom of fluorine poisoning: only the permanent teeth are affected, the milk teeth being seldom attacked. The changes are of two kinds—

(*a*) The enamel is decolorized, either generally or in

because more water is drunk there, or because the foods contained fluorine. The highest quantities of fluorine were found in mottled teeth (Smith, 1956). Since fluorine is taken up both from water and from foods, as established by Dillon (1953), quantities of 3·8 to 5·3 mg were taken up, in spite of the fact that the water contained only 1 p.p.m. of fluorine. In England, *tea contains large quantities of fluorine* from which the large amount of tooth mottling is derived. Held (1955) established the rise in blood fluorine content after administration of tablets containing fluorine; with daily doses of 5 mg the rise was *so great that inhibition of blood phosphatases* occurred.

Before fluorine is taken up by the bony tissues and the teeth, it passes from the capillaries through the capillary wall and, in fact, circulates in the blood bound to the serum albumin (Seppilli, Candeli and Scassellati Sforzolini (1957). Toxic effects first occur when large amounts have accumulated in the skeleton; for this reason, symptoms of chronic poisoning occur at a late stage.

Investigations of children in those districts in which the water has been fluoridized have so far shown no clinical signs of damage (Schlesinger, 1954; Overton and Chase, 1954; Schlesinger, Overton, Chase and Cantwell, 1955; Hoffmann-Axthelm, 1954). Vitamin C raises the deposition of fluorine in the skeleton, and in the soft tissues (Buttner and Muhler, 1957; Muhler, 1958). Linseed oil increases the toxicity of fluorine to rats. With the increase in the amount of fat, a larger deposition of fluorine in the bones became apparent, as also did a restriction in growth (Miller and Phillips, 1955). An increase in the fat content of the diet from 5 to 20 per cent is sufficient to lead to an increased retention of fluorine in the skeleton, in the liver, in the heart, and in the kidneys (Buttner and Muhler, 1958). Cottonseed oil also increases the toxicity of fluorine. The amount of cholesterol in the serum is not affected. This oil produces also an increased deposition of fluorine in the bones and in the heart, but not in the kidneys, at daily doses of 2 mg fluoride (Buttner and Muhler, 1957).

Climatic conditions apparently affect the uptake and retention of fluorine. In Australia, Crosby and Shepherd (1957) established clear differences between summer and winter; 50 per cent of the eliminated fluorine was found in the sweat. *A role in regulating fluorine metabolism* is attributed to the secretion of sweat.

Hattyasy, Straub and Toth (1956) found that caries-resisting individuals showed higher values in the autumn than in the spring and *summer. Persons with* active caries showed no seasonal fluctuations. The authors believe that the higher fluorine contents depend on the food, the water or the climate, but there may also be an endogenous regulatory mechanism.

Age *seems to have an effect on fluorine metabolism.* If rats are given 1·0 per cent of sodium fluoride for 14 days, more is laid down in those three-weeks old than in those seven-weeks old or six-months old. In the younger animals, a more marked mobilization of *fluorine is seen than occurs in the older animals* (Miller and Phillips, 1956). In contradistinction to this is the work of Ramseyer, Smith and McCay (1957); they found marked alterations in the older rats, including those of a periodontal kind and, frequently, loss of teeth as well. In the older animals they found both hypertrophy and hyperplasia of the renal tubules.

Thyroid, and the thyrotropic hormone, increase the sensitivity of the organism to fluorine (De Eds, 1941). The activity of thyroid and pituitary are important factors in determining individual sensitivity to fluoride poisoning (*see also* Jentzer, Gordonoff and Minder *et al.*).

We have taken from Dillon (1953) Fig. 9.2, which shows that carious processes can increase in persons

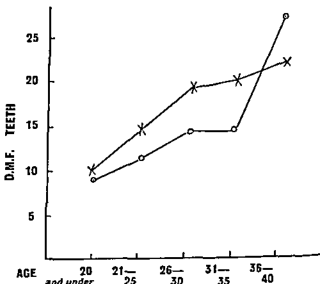


FIG. 9.2. GRAPH DRAWN FROM FIGURES OF ONE LOW-FLUORINE AND ONE HIGH-FLUORINE AREA
O——O, South Shields (0 82 p p m. F); F X——X, Ipswich (0 3 p p m. F) (quoted by *Dillon*, 1953).

more than 35 years old in spite of fluorine administration.

It seems pertinent to end this chapter on the pharmacology of fluorine by emphasizing the fact that its *therapeutic range* is extremely narrow, as is shown by the data in Table 9.4 taken from Hodge and Smith (1954).

and with addition of 16 per cent raw egg albumin, the animals lost weight, became partly bald and developed dermatitis, some of them dying. Broadly, there were symptoms of a biotin deficiency: the symptoms disappeared when the egg albumin was omitted. Control animals without fluorine remained healthy. The author is of opinion that the biotin deficiency occurs in the intestinal bacteria because of the action of the fluorine.

It has been shown by Waldbott (1955*a*) that human subjects can react with special sensitivity to the smallest quantities of fluorine. He describes a 35-year old woman with stiffness, violent pains in the spinal column, gastric disturbances, stomatites and paraesthesia in legs and hands, all of which could be traced back to the fluoridized water, in which the element was present at a level of 1 p.p.m.

It is broadly true to say that the first signs of fluorine toxication make their appearance as pitting of the teeth, though to produce this, the fluorine concentration in the water is not solely responsible, since the calcium content of the diet also plays a part. Pitting of the teeth has, in fact, been observed with as little as 0·3 mg of fluorine per litre of water.

For a searching investigation into severe fluorine poisoning we have to thank the French authors who have described the disease Darmous prevalent in Morocco. Darmous has been known for a long time, and has been described in the U.S.A. as well as in North Africa. Its aetiology was first recognized by Velu (1918), quoted by Gaud, Charnot and Langlais (1934). Darmous occurs not only in man, but also in animals, particularly in herbivores. The teeth are especially affected; they are of unusual dimensions, their shape is altered, as well as their position, and they have a peculiar colour, along with lowered resistance. Such teeth are unusually sensitive to cold. The bones are also altered, being deformed and greatly thickened. In man, the six-year molars are especially damaged (*see* Plate XIII (*b*)). Bony processes show, under X-rays, distinct osteopetrosis.

Velu established that different plants do not take up fluorine to the same extent; thus, wheat absorbs more than barley. Velu studied the uptake of fluorine by a lamb, which consumed daily: 1·25 mg in the water, 7·5 mg in mud, 33·8 mg in plants, 113·6 mg in dust. During a dry spell the animals may take up as much as 1 g of calcium fluoride in the dust.

The organism protects itself against fluorine; thus, for example, in chronic toxication little fluorine passes into the maternal milk supply. Calcium fluoride dissolves in the gastric juices, and the same is true of the bile, so that man and animals can excrete large quantities of fluorine. Protection of the organism expresses itself also as increased elimination of fluid, in the saliva, in the perspiration, in nails and hair, and as exfoliation of the skin. Charnot investigated human nails, hair and skin, parallel with those of healthy individuals, showing—

	Fluorine %
Nail	
healthy	2·9 mg
Darmous	4·9 mg
Hair	
healthy	2·0 mg
Darmous	8·0 mg
Skin	
healthy	3·1 mg
Darmous	6·4 mg

Fluorosis has also been observed in Argentina (Raffaele, 1944). In particular, it was there established that bone damage is involved, both a generalized osteosclerosis and an osteoporosis. Degenerative anomalies were found in the teeth.

From what has been written above, it will be gathered that fluorine is by no means a harmless substance. Various authors have given different values to the minimal toxic dose. The toxicity of fluorine is related to calcium intake, and to calcium metabolism generally, for the only therapy in acute and chronic fluorine poisoning consists in administering calcium. In any event, we have here an element of narrow therapeutic range.

Toxication with fluorine has been observed also in Iceland, after a volcanic eruption, and heavy poisoning in Maastal (1930) has been connected with intake of fluorine from the exhaust of factories.

VI. FLUORINE AND RICKETS

It has long been known that large doses of fluorine affect the skeletal system. Investigations on pigs (Belanger, Vizek, Lotz and Comar, 1958) have shown that administration of 1,000 p.p.m. of sodium fluoride in food produce changes in the skeletal system similar to those of vitamin-D deficiency. Knappwost (1953), on the other hand, demonstrated that small quantities of fluorine had a curative effect on experimental rickets in rats; according to his view, not only is anti-cariogenic activity to be attributed to fluorine, but "rather does it exercise a much wider protective function against degenerative processes of physiological calcium metabolism." He recommends the addition of fluorine ions to drinking water, and the administration of poorly soluble fluorides in chewing gum.

Gebauer (1956) carried out exhaustive experiments on rats and was unable to confirm the assumptions

:hes, and becomes whitish and chalk-like. This nge is already visible when the tooth erupts.

5) After eruption, there occurs, in the defective mel, a deposit of a dark pigment-like substance ch can vary from yellow to brown, or even :k. The coloured areas are seen on the labial faces of the front teeth and the eye teeth, that is, ones exposed to light.

Mottled teeth are infrequently carious, although der (personal communication) observed caries in mottled teeth of South Americans. In any event, enamel is brittle, and fillings are badly retained. surface is irregular because of either local hyposia or corrosion of the enamel. Eruption is delayed fact that has been insufficiently emphasized. The aining teeth depart markedly from the normal in , shape and position, and have a reduced resistance. teeth are also rapidly worn down and are somees painful during chewing, particularly under the uence of cold. Gingivitis and tartar deposit are quent. These teeth are to be regarded as defective ume, 1953); the enamel prisms calcify poorly. The k pigment is of organic origin and can be bleached oxidizing agents, but not permanently. The dental nges arise because the formative cells of the tooth, ameloblasts and odontoblasts, are extraordinarily sitive to fluorine. It is reasonable to assume that rine intervenes in the enzymic system of tooth cification. The lesions are irreversible. Children m 3–5 years are particularly sensitive. Since the k teeth are not affected, it is assumed that the centa acts as a barrier (*v.* Held, below). Larger ntities of fluorine (10 mg/l), however, also affect first dentition.

As fluorides have frequently led to poisoning by ident, it has been possible to study the symptomatoy more exactly.

Dyrenfurth and Kipper (1925) found, in a case of soning, first nausea, then heart failure, which they ociated with the decalcifying action, since treatnt with lime led to an improvement. One often erves, also, the early stages of nephritis.

Brinch and Roholm (1934) record an infant that l, during suckling, absorbed fluorine from a female olite worker. They were able to establish an umulation of fluorine in the bones, and its slow eration even after the end of fluorine intake.

Kilborn, Outerbridge and Hai-Peng-Lei (1950) und, in Chinese subjects, a characteristic arthritis ngside pitted enamel, with sometimes complete fening of the limbs. Water was found to contain orine, 6 p.p.m. The disease occurred in most of the oulation between the ages of 10 and 15; acute cases ld lead to death at as early an age as 18. In chronic es the patients are completely incapable of work by the thirtieth year. A Chinese subject died as a result of fracture of the spinal column, and his bones could be more closely studied. Except for the mandible, all the bones were thicker than normal by about 50 per cent, the clavicle and scapula by as much as 250 to 290 per cent. The spinal column was completely stiffened. The fluorine content of the bones was twenty times that of normal bones.

Largent (1952) was able to establish that fluoride storage for months, or even years, could follow daily oral intakes of 3 mg sodium fluoride. He reports the poisoning of a young Texan, in whose neighbourhood the fluorine content of water varied from 2·4 to 4·4 p.p.m.

Takamori and Tokushima (1955) observed, on 100 children, that the incidence of pitted enamel increased inversely with body weight. In districts with drinking water containing 1·9 to 4·8 mg/l myocardial damage and ventricular enlargement were revealed by the electrocardiogram. They tackled the matter experimentally and were able to show, in rabbits, a reduction in ST, no effect on T and an increase in QT. In the rabbit, degenerative alterations of the myocardium were revealed histologically.

The toxicology of fluorides has also been studied experimentally. Peters (1954) established cardiac disturbances in the rabbit; the dog, on the other hand, reacted with neurological disturbances.

Briggs and Phillips (1952) observed the toxic effects of fluorine in rabbits that had received from 0 021 per cent of fluoride, upwards, in their diet for four months. The first signs of toxication appeared in the teeth, but disturbances in growth later became manifest, with stiffening of the limbs and changes in the bones.

Held and Grasset (1954) treated guinea-pigs and pregnant rabbits with solutions of sodium fluoride. The animals received 10–30 injections of 1 to 1·5 mg sodium fluoride per kg daily. No signs of illness were observed, or any disturbances in pregnancy or liver, kidneys and adrenal glands. However, there was degeneration of the osteocytes and signs of atrophy in the bones, the incisor also being abnormal. Although Miller and Phillips (1955) observed that the growth-inhibiting effect of 0·1 per cent sodium fluoride in the food was increased by raising the fat content from 5 to 15 per cent, Buttner and Muhler (1958) found, in rats, that 20 per cent of fat in the diet had no effect on the toxicity of fluorine as judged by increases in weight.

Carr (1954) fed rats on a diet of casein 18 per cent, cane sugar 75 per cent, malt 4 per cent and 3 per cent of a salt mixture, along with various vitamins, and was unable to establish any appearances of ill-health from drinking water containing 80 mg fluorine per litre. However, with the casein reduced to 10 per cent,

and with addition of 16 per cent raw egg albumin, the animals lost weight, became partly bald and developed dermatitis, some of them dying. Broadly, there were symptoms of a biotin deficiency: the symptoms disappeared when the egg albumin was omitted. Control animals without fluorine remained healthy. The author is of opinion that the biotin deficiency occurs in the intestinal bacteria because of the action of the fluorine.

It has been shown by Waldbott (1955*a*) that human subjects can react with special sensitivity to the smallest quantities of fluorine. He describes a 35-year old woman with stiffness, violent pains in the spinal column, gastric disturbances, stomatites and paraesthesia in legs and hands, all of which could be traced back to the fluoridized water, in which the element was present at a level of 1 p.p.m.

It is broadly true to say that the first signs of fluorine toxication make their appearance as pitting of the teeth, though to produce this, the fluorine concentration in the water is not solely responsible, since the calcium content of the diet also plays a part. Pitting of the teeth has, in fact, been observed with as little as 0·3 mg of fluorine per litre of water.

For a searching investigation into severe fluorine poisoning we have to thank the French authors who have described the disease Darmous prevalent in Morocco. Darmous has been known for a long time, and has been described in the U.S.A. as well as in North Africa. Its aetiology was first recognized by Velu (1918), quoted by Gaud, Charnot and Langlais (1934). Darmous occurs not only in man, but also in animals, particularly in herbivores. The teeth are especially affected; they are of unusual dimensions, their shape is altered, as well as their position, and they have a peculiar colour, along with lowered resistance. Such teeth are unusually sensitive to cold. The bones are also altered, being deformed and greatly thickened. In man, the six-year molars are especially damaged (*see* Plate XIII (*b*)). Bony processes show, under X-rays, distinct osteopetrosis.

Velu established that different plants do not take up fluorine to the same extent; thus, wheat absorbs more than barley. Velu studied the uptake of fluorine by a lamb, which consumed daily: 1·25 mg in the water, 7·5 mg in mud, 33·8 mg in plants, 113·6 mg in dust. During a dry spell the animals may take up as much as 1 g of calcium fluoride in the dust.

The organism protects itself against fluorine; thus, for example, in chronic toxication little fluorine passes into the maternal milk supply. Calcium fluoride dissolves in the gastric juices, and the same is true of the bile, so that man and animals can excrete large quantities of fluorine. Protection of the organism expresses itself also as increased elimination of fluid, in the saliva, in the perspiration, in nails and hair, and as exfoliation of the skin. Charnot investigated human nails, hair and skin, parallel with those of healthy individuals, showing—

	Fluorine %
Nail	
healthy	2·9 mg
Darmous	4·9 mg
Hair	
healthy	2 0 mg
Darmous	8·0 mg
Skin	
healthy	3·1 mg
Darmous	6·4 mg

Fluorosis has also been observed in Argentina (Raffaele, 1944). In particular, it was there established that bone damage is involved, both a generalized osteosclerosis and an osteoporosis. Degenerative anomalies were found in the teeth.

From what has been written above, it will be gathered that fluorine is by no means a harmless substance. Various authors have given different values to the minimal toxic dose. The toxicity of fluorine is related to calcium intake, and to calcium metabolism generally, for the only therapy in acute and chronic fluorine poisoning consists in administering calcium. In any event, we have here an element of narrow therapeutic range.

Toxication with fluorine has been observed also in Iceland, after a volcanic eruption, and heavy poisoning in Maastal (1930) has been connected with intake of fluorine from the exhaust of factories.

VI. FLUORINE AND RICKETS

It has long been known that large doses of fluorine affect the skeletal system. Investigations on pigs (Belanger, Vizek, Lotz and Comar, 1958) have shown that administration of 1,000 p.p.m. of sodium fluoride in food produce changes in the skeletal system similar to those of vitamin-D deficiency. Knappwost (1953), on the other hand, demonstrated that small quantities of fluorine had a curative effect on experimental rickets in rats; according to his view, not only is anti-cariogenic activity to be attributed to fluorine, but "rather does it exercise a much wider protective function against degenerative processes of physiological calcium metabolism." He recommends the addition of fluorine ions to drinking water, and the administration of poorly soluble fluorides in chewing gum.

Gebauer (1956) carried out exhaustive experiments on rats and was unable to confirm the assumptions

of Knappwost. Fluorine, in the form of sodium fluoride, in various doses, was unable to influence either the initiation or the healing of rickets in rats.

Effinger (1957) explains the different finding of Knappwost and Gebauer as due to the fact that the latter used freshly-prepared sodium fluoride solutions, which have only a slight effect on the deposition of normal bone material, whereas the former carried out his work with stored solutions of sodium fluoride, in which sodium silicofluoride is formed through reaction with the walls of the glass vessel. This compound is said to have antirachitic properties. Knappwost suggests the possibility that silicon works in the organism as a mean of transporting the fluorine ion.

Parallel with the work outlined above, fluorine in small amounts has been recommended as a curative agent in bone fracture (Callam, 1933). This recommendation has not found favour, since no one would dream of treating rickets with fluorine, so we shall not discuss this matter further.

VII. CARIES

Before considering the position of fluorine as an anticariogenic agent, it is necessary to ask what precisely caries is. Galenos (131–201 A.D.) recognized caries and expressed views about its aetiology: "deficient nutrition makes the teeth weaker, brittler and softer." There have always been individual appearances of caries as human disease. In Euler (1948) we find extensive material from the end of the stone age onward. For Silesia, the frequency of caries in permanent dentition was considered to be 1·75 per cent, for milk teeth, 0·75 per cent. Today the figures range round 80 per cent. According to Euler there is an explanation for the general development of caries during the last 4,000 years in Silesia. There is an increase in caries of the milk teeth as a result of faulty nutrition of the mother during pregnancy, also of mistakes in the feeding of infants; much caries in the permanent teeth is a result of carious milk teeth at the time of changing dentition; whereas, formerly, elderly people were subject to caries, today it is extending among infants. Euler recognizes a relation between race and caries, as well as one between caries and individual constitution. He quotes the work of Nachtsheim, according to whom, in Formosa, there is 1 to 11 per cent of caries among the Malays, 40 per cent among the Chinese and 60 per cent among the Japanese. However, in this connexion one must not forget—and we shall return to this matter—that these peoples, besides having been influenced in various ways by their cultures and civilizations, feed differently. As a footnote to this we may note the report of Seith (1958), that caries is widespread among the white people of South Australia, whereas it is unknown among the natives, who continue to follow their primitive pattern of living. As is well known, there are two kinds of enamel caries, for it is the enamel that is of primary importance in this condition: the superficial frontal disturbances of the enamel, the result of a direct decalcification, and a more deep-seated process, sometimes without any clear external signs (Held, 1955). Dentine caries begins under the enamel and spreads along the enamel–dentine boundary.

Viewing current opinions about the aetiology of caries, we note that Kantorowicz (1956) holds that we are dealing here with the involvement of vitamin D. The cause of caries is attributed to occurrences at the age of enamel building, that is, in the embryo, and the first years of life during the eruption of teeth. Metabolic disturbances during this period, particularly intestinal disease, and above all rickets, disturb normal enamel development during the first and second year and make it susceptible to caries. Nevertheless, it is not impossible for a rachitic child, after its recovery, then to lay down normal enamel and develop a healthy dentition. This view of Kantorowicz can also be combined with Miller's theory: according to this, carbohydrates, acid-forming bacteria and saliva are responsible for the origin of caries. Kantorowicz was able to show that, during the nineteenth and twentieth centuries in industrial towns, the number of diseases of sucklings, in particular rickets (bottle feeding), also the tendency to caries, increased, and that increased resistance to caries resulted from preventative measures taken against rickets.

Egyedi (1958) summarizes current theories of caries—

1. The decalcification theory, involving acid formation in the plaques, and decalcification of the enamel as the primary event.

2. The proteolytic theory involving penetration of bacteria and disturbances in the organic portion of the enamel as the primary phenomenon, with secondary demineralization.

3. The glycogen theory, apparently a synthesis of the two above.

Almost all investigators are of opinion that the disturbances in the enamel can be brought about only by acid. Bacteria appear as the sources of acids. This theory, that caries is due to acid production by bacteria, is the most prevalent of all. It is interesting to note that it occurred among the older generations of dentists. Thus, we read that at a sitting, in 1867, a medical society in Berlin heard a Mr. Leber on the

ision of opinion about the origin of dental caries. ne believed that chemical agents were involved, ıve all, acids. Others spoke of parasitic action *ptotrix buccalis*). Leber maintained that tooth mps could also become carious. He argues for the naging effect of acids, upon which is superimposed action of *Leptotrix* at the damaged sites.

That saliva can play a part is easy to understand. ırdonoff (1943) and Gordonoff and Walthard (1944) nonstrated, probably for the first time, that inhibi-n of salivary secretion could produce pathological ınges in the oral cavity. Extirpation of the salivary nds could give rise to paradentites in rats. The cro-organisms undoubtedly play an important part this. Thus, one could witness the production of perimental caries in animals whose environment ntain bacteria, but not in those in a bacterium-free vironment (Eggers-Lura, 1955). Certainly the pro-ction of acid in the mouth cannot be responsible one, for it is rapidly eliminated, and the fall in pH er the introduction of glucose is neutralized in a v minutes. Saliva contains a buffer system, as well enzymes that rapidly degrade acids. To quote ;gers-Lura, who maintains that the previous view clinicians, according to which caries is a deficiency sease, is wrong: "now we know that the utilization d complete combustion of nutrients are tied up th resistance to caries and that caries is to be garded as a disease of excess. Wartime statistics ve *shown that food rationing, proper food utiliza-*on and resistance to caries always go hand in hand." 'e have already quoted earlier the work of Roos, 10 was able to show that caries was related to ıanges in nutrition, both during and after the war.

Hungerbühler (1955) made a statistical survey of hool children, aged 7–14 years, in St. Gallen, and tablished the frequency of caries during the war :ars, with a minimum in 1947, and an increase in equency since then. He holds the view that wartime ets were poorer in cereals (from 20–30 per cent), in ıgar, flour products and rice (50 per cent or more).

Driak (1956) is also firmly of opinion that nutrition lays a dominant role in the aetiology and pathology f caries, and he insists that caries first begins to ssume importance with increasing "civilization" and fined food. High levels of culture are inevitably tied p with a high incidence of caries. Although most uthors hold the views about the aetiology of caries, ımmarized above, there are others with different ıeories. But all investigators agree on one thing: that aries is not a fluorine deficiency disease (Adler, 1950). Ve have already indicated in several places that a rotective action against caries is not to be denied to uorine. Most interesting experiments were carried ut by Cheyne (1940). He used rats, fed on the Hoppet–Webber–Canniff diet, and removed the salivary glands from some of the animals; he established that the number of carious teeth to be seen in normal animals was on the average 3·5, whereas the value was 10·5 after extirpation of the gland. The addition of 0·3 mg fluorine per day per animal reduced the incidence to 2·3 in the operated rats.

Many people have been concerned with the problem of fluorine becoming effective. First, there is the explanation that the fluorine is laid down in the hydroxy apatite; during the formation of the apatite, that is, while the enamel is developing, several fluorine ions can be taken up, resulting in a fluor hydroxy-apatite in the enamel, whose resistance is thereby increased. Pure fluorapatite is a quite hard and resistant substance. For this reason attempts have been made to incorporate in the apatite crystals as many fluorine ions as possible, either by oral administration or, alternatively, by bringing the superficial layers of the enamel into contact with fluorine compounds. Yet, whether the addition of fluorine can, in fact, increase the number of fluorine ions in the apatite crystals and therefore the stability of bone minerals is not really established. Support for this view is sought in the American statistics of which we have taken note: "The statistics are assailable at many points and cannot be considered an objective indication of the successful fluoridization of drinking water" (Rheinwald, 1955). Baume (1953) has closely *studied conditions both in Switzerland and in the* U.S.A., and "reviews the many mistakes and variables which are apt to endanger the validity of statistical results and which have led, in many of the innumerable articles published on the fluorine problem, to misleading conclusions. Results in caries reduction by local fluorine application range from 0 per cent to 56·6 per cent according to the different authors."

It is remarkable, however, that the enamel takes up less fluorine than does the dentine (McClure and Likins, 1950). If water contains 0·2 to 0·3 p.p.m., the enamel contains 0 01 per cent fluorine, and the dentine 0 023 per cent; with water containing 1·0 to 1·1 p.p.m., the corresponding values are 0·013 per cent and 0 038 per cent. Contrary to the findings of other authors, McClure (1939) could not detect more fluorine in the non-carious than in carious teeth of the same individuals. It should also be emphasized that any reduction in caries brought about by the fluoridization of the water is accompanied by an increase in mottled teeth, an early sign of fluorosis (Parfit). Rheinwald (1955) observes that "only fluorine medication that goes to the verge of fluorosis has an expectation of successfully reducing the incidence of caries." Nor must it be forgotten that teeth containing fluorine,

though indeed harder, are still subject to caries when other factors are favourable (Prader, 1951).

In Section X, on fluorine and calcium, we shall review the problem of the incorporation of fluorine in the apatite lattice. We can, nevertheless, make a contribution to this problem, since we have been able to indicate that fluorine is indeed *incorporated*. *It* is, however, another question to which we do know the answer, whether under physiological conditions the possible amounts of fluorine are sufficient to increase the resistance of the dental tissue.

Lukomsky (1941) concerned himself with the local application of fluorine by brushing it on. He treated school children by brushing one-half of the tooth with the material containing fluorine and saw no caries on this side, whereas carious processes were found on the untreated sides of the molars. This account, interesting though it may be, is lacking in logic and we shall not consider it further.

Hauschild (1956) believes that fluorine ions are liberated from the fluorapatite and act bactericidally. However, we know that fluorine in fluorapatite is not exchangeable; even if it were, we also know that it is impossible to sterilize the oral cavity. Weissbach (quoted by Gordonoff) was able to establish only a transient reduction of micro-organisms by 50 per cent after carefully washing the oral cavity with alcohol swabs. One could not expect any marked effect from the small amounts of fluorine.

Among others, Sepilli, Levi and Fontana (1949) have attacked the problem of the lactobacilli and the effect of fluorine on them. It was clearly established that there is, in fact, no bactericidal action of sodium fluoride on the lactobacillus. There is a clear bacteriostatic action, but with no practical significance. It is therefore necessary to abandon the view that the lactobacillus, and indeed any other micro-organisms, can be affected by the possible quantities of fluorine appearing in the saliva.

The view of Robison (1923) that fluorine could act through its effect on the acid phosphatases, seems also to be untenable, for Euler and Eichler (1940) found fluorotyrosine to have a protective action against caries, although it has no effect on the acid phosphatases. In spite of these facts, Kleiner (1954) suggests "the mechanism is supposed to be that either the fluoride actually imparts to the tooth-structure caries-resistant properties, or it inhibits bacterial action on food particles and on dental tissue. Perhaps both occur." Thus, modern science is unable to provide an explanation for the protective action of fluorine against caries. There is much evidence that general conditions, above all nutrition, play a dominating part in the development of caries. Evidence for this is also to be found in the fact that James and Parfitt (quoted by Gordonoff) saw an effect on the teeth after acid tonics. This opinion is also in accord with the Vipeholm investigation, referred to on p. 228, and also with the beautiful work of Roos (1950) who has established a predominating role of carbohydrates, and particularly of those that remain adhering to the teeth, in the genesis of caries. The question how fluorine works, remains open. About the time at which fluorine can be effective, most authors more or less agree. Rebel (1957) says that the fluorine uptake must be endogenous, and should take place at a time corresponding with the first mineralization of the teeth, that is, for the milk teeth about the third month of pregnancy, and for the remaining teeth at birth. The same view is held by Forrest and Parfitt (1951), who also write "the ingestion of fluorine is to have an effect on the teeth, when it takes place during the period of tooth formation during the first eight years of life."

Jenkins (1954) calls special attention to considerations that should be taken to heart. He says that it is not yet certain whether or not the influence on caries is of a lasting nature, or temporary, so that the development of caries is merely postponed. Most observations have been made on children, and the few results available from adults indicate that the good effect does not last. Rebel (1957) calls attention to the need for additional medication when the drinking water contains 0·5 mg fluorine/l or more, because of the danger of a rapidly-accelerating fluorosis of the teeth (disturbances in mineralization, discoloration, hypoplasia) and a general fluorosis. It is not yet clear that chronic uptake does not lead to later damage becoming evident in the 'teens. There are proposals, in the literature, to treat mother and child with magnesium, to counteract excessive uptake of fluorine and consequent dental fluorosis (Wohinz, quoted by Rebel).

Proell (1956) expresses an opinion, to which we can fully assent, "whether or not the prominent and much propaganded protective action of fluorine will bring the hoped for 'Victory over Caries,'* as is widely thought, remains at present unsettled. The opinion that the hard tissue of the tooth can be positively affected in structure by extensive fluorine administration during pre-natal and post-natal development must be accepted, but less clear is the effect of fluorine on the fully-developed tooth. Against the elementary optimism about the advantages of fluorine medication stand the facts that the figure for successful reduction in caries in children, on the average, lies between 30 and 40 per cent, and that there are wide differences between recorded results."

* Reference to the brochure by Drum (1953), "Sieg uber die Karies."

[...]taire once said, that a doctor treats an unknown [...]e by administering an unknown drug. How apt is this remark, after 200 years, about the treatment of caries with fluorine.

VIII. STATISTICAL EVALUATION OF FLUORINE AS AN ANTI-CARIOGENIC AGENT

[...] the introduction of fluoridized water to prevent [...]s, there have been widespread statistical studies [...]nditions in the districts before and after fluori[...]ion as well as in districts differing in the amounts [...]orine present in their drinking waters. In every [...]try, if not in every city, there have been statistical [...]ngs, which have been published, first showing [...]r cent of successful results, later 50 per cent and, [...]cent times, values of 30 to 40 per cent, the last [...]ng now become a "magic number" with which [...] investigator tries to compare his own figures. [...]t quoted of all is the study at Newburgh-Kingston, [...]t which there has been much argument.

[...]e will not try to discuss these statistical findings, [...]use this would go far beyond the scope of this [...]le. It must be mentioned, however, that they have [...] subjected to very severe criticism by different [...]ors, even in the U.S.A.

[...]illon (1956) criticizes the statistical comparison [...]een Grand Rapids and Newburgh; he writes "the [...]er cent reduction in tooth decay was obtained [...]ugh grave statistical errors." Similarly Paluev [...]7) of the Statistical Department also calls attention to numerous mistakes in the statistical evaluations of the results in Newburgh and Grand Rapids.

Sutton and Amies (1958) write of the statistical investigations whose results have been published in the U.S.A.:

> "It is possible that a case for fluoridation can be solidly based, but until adequate statistical treatment of all the pertinent factors has been carried out—and this would be quite a major undertaking—the question should not be regarded as settled. In the meantime, claims concerning the amount of caries reduction are open to doubt."

Palfer-Sollier (1957) has carried out searching statistical investigations in France. He also has no doubt that fluorine can be credited with a certain activity against caries, but his quantitative conclusions are quite different.

> "Since caries, as is universally recognized, is not a disease of fluorine deficiency, there seems to be no sense in seeking methods of application based on exact statistical findings, especially since it is well known that 'with statistics you can prove anything.' "

IX. CARIES AND CARBOHYDRATES

[...]pite the possible favourable effect of fluorine [...]plementation on caries, all authors agree that caries [...]ot a fluorine-deficiency disease. Two most im[...]tant experiments, and mass experiments at that, [...] be cited as evidence that the carious processes [...] not to be traced back simply to fluorine metabo[...]

[...]wo towns on the Nile lie close together, Khartoum [...] Omdurman. The *former* has been europeanized, [...]reas the other has remained purely an Arab city. [...]e (1936) found 55 per cent of caries-resisting [...]ldren in the European town, but 94 per cent in the [...]bian. We are indebted to Roos (1950) for a highly [...]resting study. He investigated a district in the [...]nton of Valais (Wallis) that is cut off from the main [...]ffic routes. The bread there, in accordance with the [...] custom of the Valais, was baked in a communal [...]kery for the whole commune, two or three times a [...]r, and mainly from dark flour. The bread was eaten [...]rd. One of the tasks falling to the father of the [...]nily was to cut the bread with his sword. After the [...]rka railway had been built, a baker settled there, [...]d from him the population could get fresh bread daily. Instead of the old black bread, new white bread appeared on the table; along with this, caries showed a conspicuous increase.

During the 1939–45 war, the people of Switzerland ate a strong dark loaf of finely-milled flour, and both sugar and chocolate were rationed. Simultaneously the incidence of caries fell off. When rationing ended, the caries curve soon began to rise rapidly.

Both these experiments *show with all necessary* clearness that caries is basically a nutritional problem. Naturally, there are other factors to take into account, some of them of a familiar nature, and, most important among them, heredity. We have also to reckon with hormonal factors, as witness the caries of lactation; again, during pregnancy the marked tendency to gingivitis and to oedematous swelling of the gums can exacerbate pre-existing caries or even initiate it. Caries of this kind certainly has an endocrine origin.

In contrast, most caries is to be classified as nutritional caries. The human relics in Denmark (Svardborg) from between mesolithic and neolithic times, that is, between 10,000 and 15,000 B.C., showed no

caries, according to Berlin (1956). Later remains were investigated by Euler (1939), who was able by then to establish the presence of carious processes, showing clearly that the food of these primitive peoples had changed. Of especial interest are the relations with carbohydrate, and in this connexion we will consider the Vipeholm studies in a little more detail.

In the *Vipeholm Institute at Lund (Sweden)* a thorough study of the inmates led to significant conclusions. It was established that sugar consumption raised the level of caries, and that the hazard increased in proportion to the stickiness of the sugar, particularly when this was taken between meals. It is true *that there were individual differences in the incidence* of caries, but this always increased with raised sugar consumption; as soon as sugar was forbidden, the tendency to caries disappeared. Caries can arise even from a low sugar consumption (Gustafsson, 1955). The different sugars show different degrees of cariogenic activity, fructose being the most potent. *Manifestly, the daily intake of sugar in ordinary solid or liquid foods at the main meals does no damage*, because chewing at meal-times has a cleansing effect. Sugars taken alone between meals, especially sticky forms, adhere to the teeth for a long time and exert a cariogenic action.

X. FLUORINE AND CALCIUM

In 1952, we carried through experiments with ^{45}Ca. We made a calcium lactobionate containing radiocalcium, and we used this also in some later experiments. Of sister rats, one group received, as control, only the lactobionate, the other had, as well as the *lactobionate, a daily addition of 1 drop sodium fluoride* 0·5 per cent solution. Pairs of rats were killed at various intervals, and the radioactivity of bones and teeth were measured. The results are shown in Table 9.5.

TABLE 9.5

Percentage of Calcium Laid Down in Teeth and Bones

(*From* Gordonoff and Minder)

	After 12 days		After 20 days		After 28 days	
	I	F	I	F	I	F
Without fluorine	0·86	1·07	1·26	1·34	1·93	2·22
With fluorine	0·32	0·65	0·90	1·24	1·34	1·76

I = incisor. F = femur.

We have been criticized on the grounds that this antagonism between calcium and fluorine has been demonstrated with far too large and toxic doses of the latter element. This criticism is unjustified; apart from the fact that our experiments were meant to be a contribution to the fundamental study, the rat has, relatively, a much larger surface than man, and a much shorter life-span. In general, rats require fluorine in larger doses, and its anticariogenic activity is evinced in rats only after a dose of about twenty times that required *by man; for the latter 1 p.p.m.* is recommended, for the former as much as 20 to 30 p.p.m. In any event, our experiments have established what has been long known to the chemist, namely that the two elements, fluorine and calcium, act antagonistically towards each other in living beings. What the chemist has known as a result of his *experiments in the test-tube, we were able to establish in vivo*.

Our results have been confirmed by other workers. The first investigator to give information about the relationship between calcium and fluorine was Roholm (1936), who described cases of osteosclerosis, and osteomalacia, among cryolite workers, and called special attention to the fact that cattle, grazing in the *neighbourhood of aluminium factories, suffered from* osteomalacia. Roholm explained the toxicity of fluorine as due to calcium precipitation, for the disease was similar to oxalic-acid poisoning. He also expressed the view that fluorine might influence calcium metabolism via the parathyroid glands.

The experiment of de Senarclens (1941) went more thoroughly into this antagonism. This investigator made experiments with rats, rabbits and sheep, which he poisoned with quantities of sodium fluoride ranging from 3 to 10 mg. No changes were seen macroscopically, but, *according to the rapidity of the toxication*, microscopic pathological changes were observed similar to those of an osteopathy of the ostitis fibrosa type. There was first a disturbance of the bones (osteoclasis), similar to the progressive bone atrophy of Askanasy. In the second phase, bone regression of the osteosclerotic type was observed. The more soluble *sodium fluoride showed greater toxicity than* calcium fluoride. In rats and rabbits, also, a marked lowering in phosphatase content resulted.

McCollum, Simmonds, Becker and Bunting (1925) found marked changes *in the incisors and osteoporosis* of the mandibles after large doses of fluorine. Genêt (1932) carried out long-term feeding of fluorides to guinea-pigs, and established a lengthening of the incisors and a disturbance in enamel formation.

Similar changes were also seen in young guinea-pigs whose mothers had been treated with fluorine. The author calls attention to the probable toxic effects of fluorine on a child whose milk has been preserved with sodium fluoride. The enamel would be of poor quality and the teeth could become carious. Fleming (1953) found that the presence of fluorine brought about a retardation in the calcification of enamel and dentine, as well as an alteration in structure of the ameloblasts. He believes that fluorine acts on enzyme systems. Further, Fleming and Greenfield (1954) found that, after the administration of fluorine, "Calcification of bone in the jaws was retarded. There was alteration of the cell structure of the ameloblasts, with retardation of the enamel matrix maturation. Cartilaginous-like formations appeared in the dental pulp. There was a bridging of the dentine matrix formations."

Bishop (1936) established that "fluorine has no important useful biological function" and is not present in the new born; it is taken up with salt and water by the organism, and is not completely eliminated. That, in fact, not all fluorine is eliminated he established in a dead labourer who had worked in a superphosphate factory; compared with the normal, his bones showed an accumulation of fluorine. Paff and Boyd (1952) added sodium fluoride to bone cultures from chick embryos and observed a disturbance in mineralization. The inhibition by fluorine could have taken place either enzymatically or as a result of calcium fluoride formation and a consequent inadequate supply of calcium for calcification. McClure and Mitchell (1931) found no bone changes in rats on a diet containing 0 01 per cent of fluorine, but 0 03 per cent produced such changes with calcium loss.

It is obvious that changes are most pronounced in workers involved in the breaking up of cryolite. Flemming Møller and Gudjonsson (1932) noticed, in such workers, sclerotic affections of the bones, banding, and muscle joints, which they seek to relate to the deposition of calcium fluoride. The gastric mucosa of these patients showed erosion, which the authors attribute to the conversion of the cryolite dust into hydrofluoric acid by gastric hydrochloric acid. Jackson (1955) administered fluorine for six months from birth onwards. The fluorine content of the bones increased with age, and fluorine retention was the same whether he gave a single large dose or smaller doses spread over a period. With 1 mg/100 g 35 per cent of fluorine was retained, and fluorine retention could be reduced only by giving higher doses of calcium. Similarly, Cremer and Voelker (1953) observed a rapid loss of weight in rats receiving 0·1 per cent fluorine as sodium fluoride, and the animals deteriorated; on addition of 3 per cent calcium nitrate, recovery set in. Zipkin and McClure (1952) found that rats receiving large quantities of fluorine showed an increase of fluorine in the bones up to 200 days old, whereafter the values remained constant.

The damage observed with larger doses of fluorine have frequently led to views that, like those quoted above from Genêt, may serve as a warning against fluorine. Thus, Box (1955) writes: "Fluoridated water is condemned, because it is harmful to gingival or periodontal tissues. Fluoridation seems to promote the growth of buccal algae and fungi and to cause deposits of calculus. In areas with fluoridated water a high incidence of gingivitis is found."

Characteristic changes are clearly seen only after larger doses. Zipkin, McClure, Leone and Lee (1958) observed uptake of fluorine in bones, which was linear, only after 4 0 p.p.m. At Colorado Springs, where the water contains 2·5 p.p.m. of fluorine, Geever, Leone, Geiser and Lieberman (1958) found no pathological bone changes.

The antagonism between fluorine and calcium is also expressed as an increased elimination of calcium. Flück (1955) investigated the effect of 1·25 mg of sodium fluoride per litre of drinking water on calcium elimination in the milk of a goat, using for this purpose ^{45}Ca. Over a period of 21 days he found that the goat eliminated twice as much calcium in the presence of fluorine as in its absence (*see* Fig. 9.3). It took another two months for radiocalcium to be no longer demonstrable in the milk, whereas without fluorine the labelled calcium was almost completely eliminated within three weeks.

The antagonism between fluorine and calcium was also observed in the findings of Loewe (1934). He established by X-rays the presence of rickets, with poor calcification in the zones of ossification and a widening of the epiphysis, after fluorine treatment.

It is understandable that this antagonism has led to the view that there must be some relationship between the action of fluoride and the parathyroid glands. Gershmann (1930) produced hypoalkaemia in dogs by intravenous injections of sodium fluoride. The calcium content of the serum rose after three hours, but without reaching the normal value. If thyroids and parathyroids are removed from the dog, it is no longer able to undergo compensatory hypoalkaemia.

Pavlovic and Bogdanovic (1932) found, in a fluorine poisoned rabbit, cellular enlargement of the parathyroids, with hyperaemia and capillary dilatation, as well as haemorrhages. In acute poisoning, degeneration of the parenchyma predominated. Kochmann (1934) made the observation that fatal oxalic-acid poisoning can be counteracted by parathyroid hormone, and that similar conditions may be seen after

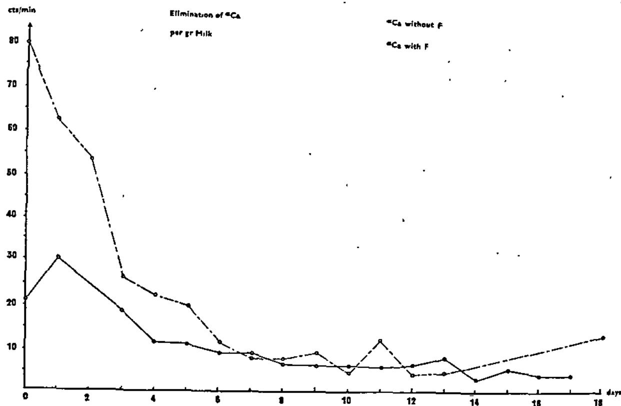


FIG. 9.3. CALCIUM ELIMINATION IN THE MILK OF A GOAT, WITH AND WITHOUT FLUORINE ADMINISTRATION

sodium fluoride; he proposed the administration of fluorine in the assessment of parathyroid hormone.

Waldbott (1957) described tetanic cramps in a 12-year old boy elicited by traces of fluorine in the drinking water.

Comar, Vizek, Lotz and Rust (1953) experimented on pigs with radiocalcium, and found a shift of calcium from the neighbourhood of the epiphysis. In their view, fluorine raises the degree of resorption in the bones. A contribution to the antagonism between calcium and fluorine also comes from Hauck, Steenbock and Parsons (1933), who fed animals on a synthetic diet containing either 1·22 per cent or 0·1 per cent calcium. Fluorine was more toxic to the animals receiving the calcium-low diet.

We do not propose to discuss this problem further. It is certain that we are confronted with an antagonism between calcium and fluorine, which becomes more and more evident with increasing doses of fluorine, although it may also appear after small doses, which can cause retention of fluorine as well as increasing the amount and rate of calcium elimination. Many observations made lead to the view that nutritional conditions can, in themselves, play a part in this antagonism. Dwellers in the Campagnano di Roma and Quarto, where nutritional conditions are bad, and the diet is poor in protein and calcium, showed an increased sensitivity to fluorine. Rats receiving increased amounts of calcium, deposit less fluorine in the bones than control animals (Largent, 1954). Day (1940) found, among Indians, an increased incidence of mottled teeth among the under-nourished. Similarly Murray and Wilson (1948) found that European children in Morocco showed less mottling of the teeth than Arabian children, though they all used the same drinking water. Although the teeth react first to fluorine, Werbeloff (1958) found no symptoms in the dental tissues of a South African coloured woman in a district with endemic bone fluorosis, in spite of bone damage so extensive as to immobilize the patient. Even with an amount of fluorine as small as 1 p.p.m. Minder and Gordonoff (1958) could establish, after three weeks, a reduction of molar calcium incorporation by 5 per cent. In the long bones and the incisors no clear changes were observed.

Irving and Ninaber (1946) fed rats on one of three diets (Table 9.6).

After four weeks the animals received, subcutaneously, 2 per cent sodium fluoride. On diets 1 and 2, amounts of 26 mg/kg were not always fatal. On diet 3, tetany

TABLE 9.6

Diet	Ca per cent	P per cent	Ca:P
1	1·23	0·84	1·5
2	1·31	0·24	5·5
3	0 098	0·37	0·26

set in immediately, and all animals died. The authors believe that an excess of phosphate leads to a "pre-tetanic" condition, which is alleviated by fluorine. Shourie (1948) was able also to reduce, or even to abolish, the toxic action of interperitoneally-administered sodium fluoride by means either of calcium or of parathyroid hormone. Krilova, Gnoyevaya and Sribner (1957) studied the fluorine contents of the teeth of rats brought up on various quantities of calcium. Their results are set out in Table 9.7.

TABLE 9.7

Calcium content of rats' diet (mg per cent)	Fluorine content of incisors after 5 mg/kg
7·1	147·8 mg %
59·3	114·7 ,,
259·3	62·6 ,,

The animals receiving diets poor in calcium reacted *more violently to fluorine, and died sooner. The* authors recommend the supply of calcium in large quantities prophylactically to the populations of fluorosis regions. A similar view is expressed by W.H.O. (1958), that diets high in calcium reduce the absorption and toxicity of fluorine.

It is well known that the nutrition of populations of various contiguous regions can differ, so that the calcium contents of their diets may differ also. We quote, in Table 9.8, from a report of the United Nations (1958) figures for the dietary calcium intake for various places.

Clearly, in such circumstances, the same amounts of fluorine could have quite different effects according to the calcium intake.

From the findings mentioned before there derives the general view that the fluorine ion is built into the apatite lattice of bone material, with a resultant dominating effect on the whole tissue of the developing tooth; moreover, it enters the apatite lattice in place of OH ions; this phenomenon is not completely

TABLE 9.8
Daily Calcium Intake (mg per Person)

Country	Cereals	Milk	Fish
Australia	52	570	—
Argentine	84	510	12
Canada	109	780	—
Japan	264	20	106
Philippines	53	32	—
South Africa	56	260	7
England	370	585	12

reversible. Neuman *et al.* (1950) established that, in bone powder, fluorine can replace OH and HCO_3 ions, but not calcium or phosphate ions. A crystal lattice structure assumed for bone mineral is based upon the formula for apatite, Ca $(Ca_3 (PO_4)_3)_2 X_2$, in which the ions designated X can be OH, Cl or F, that is, giving hydroxy-, chlorine- or fluorapatite. However, in bones, at most one-quarter of X can give fluorapatite. Fischer, Ring and Muhler (1956) established by various X-ray methods that "fluorapatite is not the dominant product in the treatment of enamel with fluorides." Perdok (1952) found by means of the Debye–Scherer radiograph that by flotation of powdered teeth in a solution rich in fluorine there was formation of calcium fluoride, which is not possible with the lower concentrations of fluorine such as occur *in vivo*. There, synthetic hydroxyapatite passes, even at room temperature, into fluorapatite.

It is well known that there are no experimental *procedures for isolating, without alteration, the in-*organic portions from fresh bone. Inorganic bone preparations are, in any event, to be taken as more or less altered artefacts. Most investigations have been carried out on bone ash. During the ashing process the highly unstable and somewhat irregular structure of the small "bone phosphate" crystals are changed to the structure of apatite, with the characteristic X-ray interference picture. At the same time there is a considerable increase in size of the crystallites. Moreover, it is also clear that the bound water disappears during incineration at the same time as the material undergoes chemical and structural changes.

It need hardly be pointed out, that to designate the crystalline portion of bone as apatite, or even as hydroxyapatite, is a gross over-simplification, though for ashed bone preparations this is broadly, but not completely correct.

The dehydration of fresh bone material occurs in three stages. In the temperature range between 110° and 600°C the bone material loses the water from the

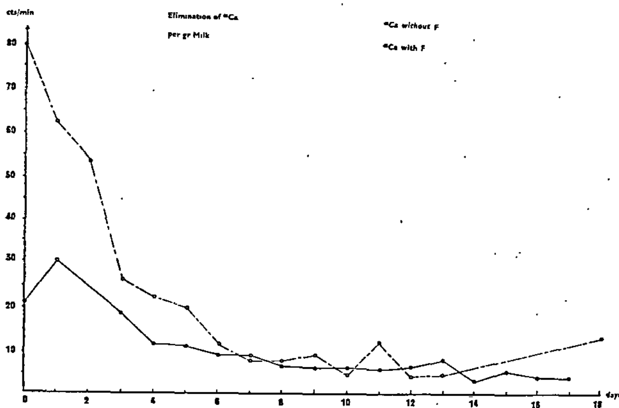


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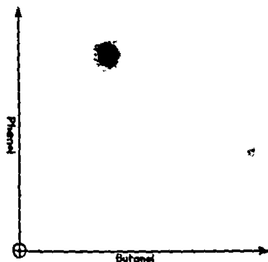
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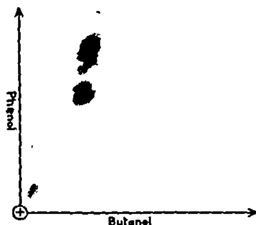
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(a) Autoradiogram of a Two-dimensional Chromatogram of Radiodiiodotyrosine



(b) Autoradiogram of a Two-Dimensional Chromatogram of Radiodiiodotyrosine Treated with F-ion. Separation into Fractions, one being Monoiodotyrosine

Control without fluorine

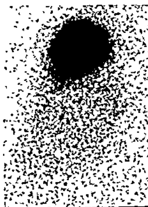




With fluorine

(c) Slices of the Hypophyses

Control without fluorine





With fluorine

Plate XIV

(d) Radioautographs corresponding to (c)

After fluorization the hypophysis is not marked.

"hydration shell"; above 850°C the hydroxyapatite loses its water also and is partially changed into β-tricalcium phosphate.



$$2\,Ca_5(PO_4)_3OH \longrightarrow 3\,Ca_3(PO_4)_2 \cdot CaO + H_2O$$

This change was clearly demonstrated for dental enamel by Carlström (1955), and for artificial precipitated calcium phosphate by Wallaeys (1952), who both used X-ray procedures. Even though Brandenberger and Schinz (1946) dispute the occurrence of this change in bone substance, we must accept the findings of Dallemagne and Brasseur (1942), who also were able to establish that, above 600°C, the bone substance undergoes an increasingly rapid change into β-tricalcium phosphate, in accordance with the equation given above. This type of change in the bone substance is also made evident macroscopically. Under 600°C the ashed bone has a *pumice-stone-like* appearance and is not markedly changed, whereas the strongly heated material is reduced in volume, being very hard and compact. The wide-angle radiograms taken by us (*see* Fig. 9.4) confirm the changes outlined above. Rat bones ignited at temperatures up to 800°C show several separate intensive interference lines that cannot be attributed to apatite; they are much more markedly present in material ignited above 900°C and belong to β-tricalcium phosphate.

In order to examine the effect of fluorine additions, at the physiological levels, on the water content of bone, a group of ten rats was given, for two weeks, a quantity of 2·5 mg sodium fluoride on week-days (total 13·5 mg of F), ten animals which received no fluorine serving as controls. The femurs of the experimental animals were dried for 48 hours at 110°, weighed, then ignited at 800°C and weighed again. The loss on ignition, expressed as a percentage of the dried femur, was for the animals receiving fluorine 66·7 (±0·9) per cent, and for the controls 61·4 (±1·5) per cent. In the bones dried at 110°C supplementary fluorine was thus found to have produced a clearly increased amount of bound water, to the extent of 8·6 per cent in our experiments. Calculated per molecule of apatite, this is almost exactly 5 molecules of H_2O.

We have also gone further into the question of apatite structure. We prepared wide-angle radiograms of the bones powdered after the dehydration described above. As already stated (*see* Fig. 8.6), these radiograms contain, besides the apatite lines, several interference lines from β-tricalcium phosphate (indicated by *). The *lattice dimensions are:*

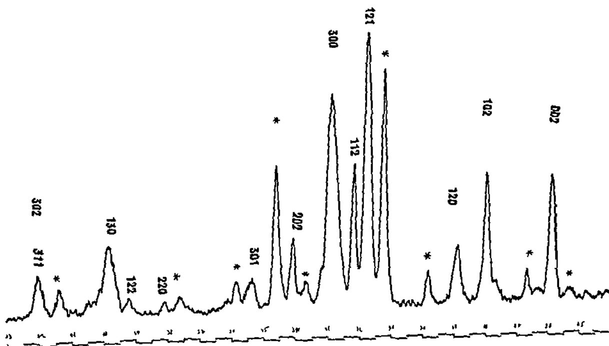
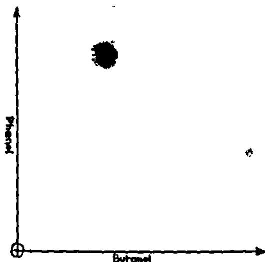
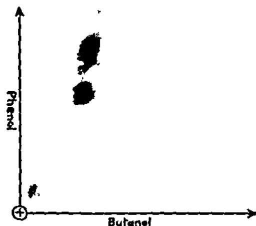


Fig. 9.4. X-ray Diffractogram of Femur Bone Material Heated at 800°C from Rats Treated with 30 mg of NaF during 2 *Weeks*

A measurable contraction of the crystallographic a_0 axis resulted by F-administration. Incinerating results in a partial conversion of apatite to -tricalcium-phosphate+CaO (lines marked with *).



(*a*) Autoradiogram of a Two-dimensional Chromatogram of Radiodiiodotyrosine



(*b*) Autoradiogram of a Two-Dimensional Chromatogram of Radiodiiodotyrosine Treated with F-ion. Separation into Fractions, one being Monoiodotyrosine

Control without fluorine

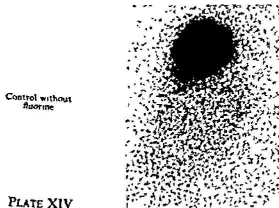




With fluorine

(*c*) Slices of the Hypophyses

Control without fluorine





With fluorine

Plate XIV

(*d*) Radioautographs corresponding to (*c*)
After fluorization the hypophysis is not marked.

Without F $a_o = 9{\cdot}403 \pm 0{\cdot}003$ Å
$c_o = 6{\cdot}865 \pm 0{\cdot}005$ Å
With F $a_o = 9{\cdot}380 \pm 0{\cdot}004$ Å
$c_o = 6{\cdot}871 \pm 0{\cdot}006$ Å

The differences in lattice dimensions (precision determinations) are small in themselves, but are certainly real for the a-axis. We have incorporated the lattice dimensions in a diagram of mass values for the mixture series hydroxyapatite–chlorapatite and fluorapatite–chlorapatite, taken from Wallaeys (1952). The apatite of bone substance from animals receiving no fluorine conforms with almost mathematical precision to the values for hydroxyapatite; for bones from animals receiving fluorine, the dimensions of the lattice lie mid-way between those of hydroxyapatite and fluorapatite. *Thus, supplementation with fluorine produces in the bones an inorganic substance that, after ignition, is clearly displaced in structure towards that of fluorapatite* (Fig. 9.5). Fluorine reacts with the inorganic substance of bone, apparently by occupying the superficial hydroxy positions, with simultaneous absorption of about 10 molecules of H_2O per fluorine atom. Whether, in fact, this amount of fluorine is sufficient to make the dental tissue as hard and firm as is generally believed, and whether, in fact, the anticariogenic action of fluorine can be explained in this way, are questions that we are not able to answer.

Röckert and Sunzel (1960) in a recently published paper ("Skeletal lesions following ingestion of fluoridated water") found quite impressive differences in the microradiograms of spine slices from rats, to which they had administered fluoridated water (20 mg and 40 mg of fluorides/l.). The calcification was found to be highly influenced by fluorine.

"No unmistakable gross radiographic lesions were apparent. The microradiograms nevertheless disclosed distinct differences between the groups receiving fluoridated water and the control group in the degree of skeletal mineralization. However, such dissimilarities were apparent only in sections from the spine and not in those from other parts of the skeleton. Normally osseous tissue includes Haversian systems with varying degrees of mineralization, as was the case in the controls. In other words, X-ray microscopically visible osteosclerosis would seem to have set in."

We have been speaking generally only of the bone substance. There is, however, a significant effect of the matrix and the action of fluorine on it. Candeli and Scassellati Sforzolini (1953) have established an affinity of the fluorine ion for mucoproteins, specifically for collagen; particularly in the mechanism for fixing fluorine do the mucoproteins play an outstanding part.

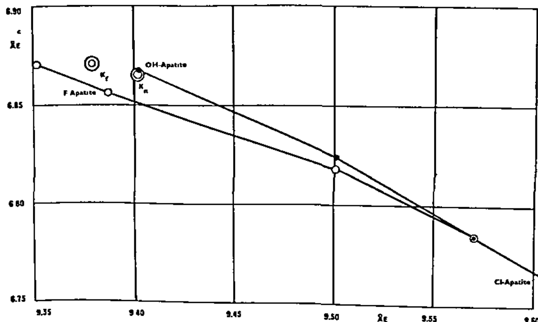


FIG. 9.5. DIAGRAMS OF THE UNIT CELLS OF ISOMORPHOUS MIXTURES BETWEEN HYDROXYAPATITE AND CHLOROPATITE (FULL CIRCLES) AND FLUOR- AND CHLORAPATITE (CIRCLES), after *Wallaeys*

Double circles—our measurements with incinerated bone material from normally-fed animals (K_n) and animals after NaF intake (K_f)

Wadhwani (1954) found a reduction in the nitrogen content of bones after fluorine administration, and the reduction was greater the poorer the diet in protein. The loss of nitrogen, after fluorine administration, was reduced if calcium and phosphorus were fed at sufficiently high levels. To both these elements a certain protective action against fluorine must be attributed. This author made (1955) similar observations on monkeys, in which he also observed a lowering of nitrogen values compared with controls. In fluorine poisoning there is a disturbance not only in the deposition of minerals, but also in the formation of fibrous tissue. This disturbance in collagen formation can even be seen when alterations in the amounts of calcium and phosphorus are not yet apparent.

The factors affecting the organic part of the bone substance have so far been too little studied, and they are highly relevant to the fluorine problem.

XI. FLUORINE AND THE THYROID GLAND

It is well known that there is a special relationship between the thyroid gland and the halogens. Thus, the main part of iodine taken into the body is trapped in the gland and used there in the synthesis of hormones. Bromine also, as we shall show below, is rapidly taken up by the thyroid. This is even more marked with astatine. It is a reasonable assumption that fluorine will behave fundamentally in the same way.

In 1854 Maumené maintained that noticeable swellings occurred in the neck of a bitch to which he had been feeding 10 g sodium fluoride daily for a period of over 4 months. He put forward the suggestion that a goitrogenic action should be attributed to fluorine and that fluorine was the goitrogenic factor in "goitre water." Since that time there are to be found in the literature innumerable references to the relationship between fluorine and the thyroid, and the frequency of goitres in regions where fluorosis is endemic has struck many observers. Attention was called to this connexion in England (Wilson, 1941) and in South Africa, in man, as well as in animals (Steyn, 1955; and Steyn and Sunkel, 1954), and in Italy (Fiorentini, Galeazzi and Visintin, 1947). Wespi (1954) studied the populations of Campagnano di Roma, of Quarto di Marano near Naples, also of Casamicciola on the island of Ischia; he was able to demonstrate a marked dental fluorosis along with a clear, though slight, occurrence of endemic goitre. On the other hand, there are not lacking indications that in some areas with endemic fluorosis, Morocco and Iceland, for example, there is no special goitre incidence.

The connexion between thyroid and fluorine has also been experimentally studied. Cristiani (1930) established that administration of sodium fluoride or fluorsilicates in large quantities led, after several months, to the appearance of signs of toxication; with smaller doses they appeared only after longer intervals. Fluorine is to be detected, above all, in bones; man and other animals living in a fluorosis district suffer from a condition that Cristiani calls *fluorose larvée*. In guinea-pigs poisoned with fluorine there have been found changes in the thyroids, in particular, proliferation of the parenchymatous tissue and, though less frequently, of the interstitial tissue. The hypophysis enlarges, and shows signs of atrophy among the cellular elements.

After Pighini (1923) and others had reported that goitres were not poor in iodine, but rich in fluorine, Goldemberg (1930) introduced ammonium fluoride for the treatment of Basedow's disease. He gave this salt by mouth, or 2 per cent sodium fluoride intravenously, and had some therapeutic success. Fluorine treatment has also been adopted with marked success by other workers, for example, by Todd (*v.* May) in London, by Coton (quoted by May) in Brussels, and by Görlitzer (1932) in Vienna. It has been shown that the administration of fluorine always brings about a lowering of the raised basal metabolism occurring in hyperthyroidism.

In Germany, May (1950) tackled Basedow's disease broadly by means of fluorine. In a large number of hyperthyroids he was able to establish a surprising improvement after 16 weeks treatment with 18–20 intravenous injections of 2 per cent sodium fluoride, an improvement such as, at that time, was never to be observed, with conservative treatment. He was also able to affect the thyroids by topical application of 10 per cent sodium fluoride ointment. As ammonium fluoride was not easily tolerated, and its intravenous injections were both damaging and, also, not entirely free of pain, May (1951) introduced fluorinated tyrosine. Fluorotyrosine was soon commercially available as the product "Pardinon" and remained for some time the preparation of choice; it was soon followed by a competitor, "Capacin"—3-fluoro-4-hydroxyphenylacetic acid—(Kraft and Dengel, 1952); successful therapy was achieved with this also. The subjective symptoms such as vegetative excitability, perspiration and diarrhoea soon diminished, and the patients were no longer troubled with palpitations and anxiety states; in 82 per cent of the cases the basal metabolic rate was lowered. At that time no other conservative treatment for hyperthyroidism was

known, so that there was much interest in the success of fluorine therapy. Experimental investigations by Litzka (1936*a*, *b*) showed a distinct lowering of the toxic dose of acetonitrile by means of organic fluorine compounds, as well as inhibition of the increased resistance produced by thyroxine. Fluorotyrosine reduces the removal of glycogen from the liver after treatment with thyroxine. Thus, there has been experimental demonstration of the antithyreogenic activity of fluorine, as well as of its antithyrotoxic action and its reduction of basal metabolic rate. Although some authors attribute these activities to fluorine as such, Litzka holds the view that fluorotyrosine acts as the whole molecule. Of later authors we would refer to von Hodenberg (1941), who was able to establish successful action on mild cases of hyperthyroidism. Manolesco (1942) obtained cures of hyperthyroidism with ammonium fluoride, and Casterra (1948) with 3-fluoro-4-hydroxyphenylacetic acid. Görlitzer (1932) successfully treated hyperthyroids with baths containing hydrofluoric acid, 30 ml of the concentrated acid per individual bath.

May (1951) was able to establish, after fluorine *administration, the appearance of a picture similar to* that occurring in "mountain thyroid"; that is, when iodine stimulates the follicles to the secretion of colloid and to become enlarged, the effect of fluorine is to reduce the colloid and to increase the supporting tissue. May summarizes (Table 9.9) the antagonism between iodine and fluorine.

TABLE 9.9

Thyroxine	Organic Fluorine Compounds
1. Accelerates glycogen liberation from the liver of the albino mouse	reduces glycogen liberation
2. Accelerates in the breakdown of muscle glycogen	reduces loss of muscle glycogen
3. Raises the amounts of glycolytic ferments in the tissue cells	produces marked reduction in glycolytic ferments
4. Produces "Basedow-like" condition in the thyroid	causes thyroid to show a microfollicular hyperplasia of the thyroids similar to "mountain thyroids"
5. Raises basal metabolic rate	reduces basal metabolic rate
6. Reduces body weight	raises body weight
7. Accelerates metamorphosis of tadpoles	0.07 mg fluorine neutralizes effect of 0.015 mg tyroxine on metamorphosis
8. Raises resistance of the albino mouse to acetonitrile	reduces the resistance to acetonitrile
9. Antagonizes insulin	increases insulin activity or replaces insulin
10. Reduces blood calcium	raises blood calcium
11. Reduces blood iodine	raises blood iodine

Görlitzer (1932) confirmed the antagonism between iodine and fluorine. Of the halogens investigated he found that fluorine and chlorine reduced basal metabolism, whereas bromine and iodine raised it. Hydrofluoric acid at a dilution of 1 in 25,000 was found experimentally to inhibit the metamorphosis of tadpoles. Euler, Eichler and Hindemith (1949), among others, studied the effect of organic fluorides on the thyroid. All fluorine preparations led to a columnar alteration in the thyroid epithelium, and sodium fluoride to a reduction of proliferation; these were only incidental findings.

Jentzer (1954), in experiments on rabbits, showed that administration of fluorine reduced metabolism and caused a reduction of thyroxine in the posterior pituitary lobe, the thyroid developing a pathological appearance.

Galletti, Joyet and Jallut (1957) investigated thyroid function in hyperthyroids treated for 20 to 267 days with 4 to 10 mg sodium fluoride. Thyroid activity was affected by sodium fluoride, but the effect set in extremely late and was not always regular. Basal metabolism, the initiation of iodine uptake by the *thyroid in tests with radio-iodine, also the amount* of iodine bound to plasma protein were significantly reduced. Of the 15 hyperthyroids investigated, 6 showed clear clinical improvement. These authors also used ^{18}F, but were unable to detect any massive storage of this isotope in the thyroid. They expressed the view that fluorine inhibits the uptake of inorganic iodine by the thyroid in a manner similar to perchlorate.

Schmid, the veterinary bacteriologist in Bern who died recently, sent us an interesting observation. In a large herd situated near an aluminium factory in Valais (Wallis) numerous calves in the years 1947 and 1948 were born with deformed legs, and, generally, gestation time was higher than normal. At first, rickets was suspected, and the cows were treated with *calcium phosphate, with little effect. In 1949* most of the calves died at birth, the rest being of poor viability and having bent legs; they could not stand, had no teeth and were afflicted with goitre. As many other animals perished, a beginning was made of treating the drinking water with a soluble inorganic salt of iodine, along with other salts. After only a few months conditions improved, and the calves were born free of goitre. Soil samples taken in this neighbourhood showed high fluorine contents (160 mg/100 g). Further, 380 mg of fluorine per 100 g of bone ash were found in the bones of an ox.

Here we have a clear indication of the antagonism between iodine and fluorine. Wilson (1941) also calls attention to the fact that an increased fluorine content of the tissues involves raised iodine utilization and can in this way lead to goitre.

We also have concerned ourselves with the antagonism between iodine and fluorine and, to that end, have administered radioactive iodine, with or without sodium fluoride, to rats. After killing the animals we established the radioactivity of the iodine taken up in the thyroid. Our results are shown in Table 9.10.

TABLE 9.10

Experiment	Duration (weeks	Number of animals	Counts per minute	
			Iodine	Iodine+NaF
I	1	4	193 (±20)	154 (±33)
II	2	10	254 (±17)	234 (± 9)
III	4	10	286 (±13)	239 (±11)

With the increase in fluorine intake, the amount of

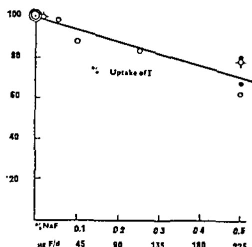


FIG. 9.6. UPTAKE OF ^{131}I IN THE THYROID GLAND (RAT) IN DEPENDENCE FROM THE AMOUNT OF F ADMINISTERED

iodine in the thyroid falls down. In Fig. 9.6 it will be seen that the level of radioiodine in the thyroid *falls linearly with the amount of fluorine administered.*

We have been criticized by certain authors for these findings. In particular, it has been alleged that we used doses which were much too large, in view of the recommendation to use 1 p.p.m. of fluorine against caries. To this we would reply, first, that we have not been concerned with specific findings, but have been carrying out fundamental investigations. We wished to know the kind of alterations which take place in the thyroid when they are submitted to fluorinization. Secondly, it must be borne in mind that the rat has a relatively greater surface area and a relatively greater metabolic rate than man. Donaldson (1924) points out that two weeks in the life of a rat are the equivalent of one year of human life. For this reason *we were justified in using larger doses for the rat.*

Our experiments have also been repeated, but by inadequate methods. Thus, for example, Demole (1954), in experiments lasting a few days, was unable *to confirm the influence of fluorine on the thyroid.* He administered radioactive iodine to rats and attempted to establish the iodine content of the thyroids in the narcotized animals by measuring the γ-rays. Our *experiments, on the other hand,* were carried out on excised thyroids. We arranged for Ruch (1958) to feed radioactive iodine to rats, with or without fluorine, and to make comparative measurements both on the *whole narcotized animal and on the excised thyroids.* Measurements on the former were carried out by means of a gamma counter; on the latter, by means of an end-window counter, which is known to give more exact results because of suitable geometry. Admittedly, in man, one is compelled to make the measurements with a γ-counter, but, in man, the relative quantities are greater. In our rat experiments *it was possible to establish a difference of 20–30 per cent* in iodine uptake as a result of the action of the fluorine; the comparative investigations of Ruch on the whole animal, and on the isolated thyroid from *the same rat, gave differences of 30–40 per cent, and* the differences between results with and without fluorine are of this order.

Levi and Silberstein (1955) withheld fluoridized *drinking water for six months from 17 euthyroid* volunteers, and measured the iodine intake in the neck region after administration of 50 μC of ^{131}I orally. The volunteers then received 4 mg of fluorine *as sodium fluorsilicate daily, for ten weeks.* The iodine uptake was again measured after three and ten weeks. No significant differences were found in radioiodine uptake by the thyroid.

Auskaps and Shaw (1955) fed various amounts of sodium fluoride to rats, and maintained that they were unable to establish thyroid hypertrophy with 1, 5 or 20 p.p.m. These authors weighed the rats' thyroids. Anyone who has ever removed the small thyroid of the rat will know how easily the preparation dries up and how difficult it is to detect small differences by this somewhat clumsy procedure.

Much more authoritative for us are the findings of Korrodi, Wegmann, Galletti and Held (1955) on humans, as well as on rats. In the former, they were unable to establish any detectable changes of thyroid after 2 mg fluorine daily. In other experiments, including among them a single sufferer from Basedow's disease, they gave 5 mg fluorine daily and were able

to establish clear changes, in which the protein iodine showed a marked tendency to fall, though without going below the normal range. In the Basedow case, an iodine reduction from 19 to 11 γ% was established. A clear reduction of thyroid activity was also established with 5 mg fluorine. The experiments with patients gave results similar to those with rats, in that a significant impoverishment in iodine of the thyroid was obtained with fluorine. Wespi (1954) also observes that "fundamentally it must be accepted that there is, up to a point, an antagonism between fluorine and iodine."

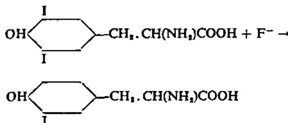
Galletti, Held, Korrodi and Wegmann (1955) specifically state "In the supplementation with doses of fluorine customary for the prophylaxis of caries, up to 5 mg fluorine can be tolerated without disturbances, provided there is simultaneous elementary intake of iodine as iodized table salt." Thus, simultaneously-administered iodine counteracts the effects of fluorine.

It has long been known that the thyroid is particularly sensitive to halogens. In experiments with radioactive bromine, Abelin and Poretti (1952) established that the thyroid takes up, at a rapid rate, not only radioactive iodine, but also radioactive bromine. The uptake of each is dependent on the functional condition of the thyroid. Hyperthyroid rats take up bromine more slowly, and in relatively smaller quantities, into the thyroid than do animals with normal thyroids.

Williams, Jaffe and Soloman (1950) established that the halogen salts, sodium bromide, sodium fluoride and sodium chloride, raise in rats the goitrogenic action of thiouracil; of these, sodium fluoride is the most active, and sodium chloride the least. The authors believe that the cells of the thyroid absorb the halogens differentially, and that re-absorbtion through the renal tubules is also differential.

An unbiased study of these experiments must lead to the conclusion that the thyroid undergoes some alteration under the influence of fluorine. To confirm this we have carried out a series of experiments, in which we have treated dibromotyrosine with equimolecular amounts of fluorine, chlorine, bromine or iodine. The chromatogram shows, with more than adequate clearness, that it is just with fluorine that changes occur. We have carried out further experiments with radioactive iodine, by treating ^{131}I labelled di-iodotyrosine with equimolecular quantities of the halogens, and preparing autoradiograms. Plate XIII(*c*) is one of these, and shows that radioactive di-iodotyrosine also undergoes some changes under the influence of fluorine. Plate XIV (*a*) and (*b*) show the effects on two-dimensional autoradiograms, in which the fluorine again has produced some changes in di-iodotyrosine.

We have investigated in detail these changes in the chromatograms (Minder and Gordonoff, 1957) and compared them with the chromatograms of mono-iodotyrosine and mono-iodomonofluorotyrosine, which we have prepared by synthesis. Conversion of di-iodotyrosine into mono-iodomonofluorotyrosine could not be established; but a partial deiodination of di-iodotyrosine to mono-iodotyrosine occurs by the action of fluorine.



Fawcett and Kirkwood (1953) shook freshly homogenized thyroid tissue with radioactive iodine, extracted the homogenate with butanol, and carried out paper chromatographic and radio-autographic analyses. Like us, they were able to establish that iodine compounds are converted by enzyme action into mono-iodotyrosine. The same enzyme also catalyses the formation of 3-fluoro-5-iodotyrosine from 3-fluorotyrosine.

Our experiments have also shown that a reduction in activity of the thyroid hormone occurs under the influence of fluorine. However, goitre need not result from this; it is possible, but not inevitable. Naturally, in the course of time, such a hypoactive thyroid might degenerate into a goitrous condition, but this need not always be so. It is true that many goitres are seen in fluorosis regions, but the whole population is not goitrous. It is necessary to distinguish between the goitrogenic action and the thyroid-inhibiting activity. Clinicians recognize, besides hypothyroidism, a condition of dysthyroidism, and it follows from our experiments that such dysthyroidism must occur, for we established that iodine uptake diminishes with fluorine intake.

In the recently-published monograph by Grab and Oberdisse (1959) the biosynthesis of the thyroid hormone is described. It is well known that the thyroid takes up iodine and retains it tenaciously. This process is described as iodination. Apparently, there takes place here, an exchange of ions by a mechanism of the thyroid that is easily demonstrated with radio-iodine. The thyroid also takes up other halogens, albeit in different proportions, but they are not retained long, nor are they built up into organic compounds; iodine alone undergoes this characteristic process. Compounds analogous with those of the

halogens; when they are physicochemically similar to iodides, such as, for example, rhodanides and perchlorates, are in a position to oust iodine from its place. Iodine is bound by the enzyme iodinase, and is then oxydized by another enzyme, iodase, and built into the phenolic amino acids, particularly into tyrosine. In this way mono-iodo- and di-iodotyrosine arise, and two molecules of the latter can condense to thyroxine. It is apparently with these processes that fluorine must interfere.

According to Grab and Oberdisse (1959) "the influence of fluorine in suitable doses on the thyroid is incontrovertible." It is not yet clear how this influence is exerted. In any event, the thyreotropic hormone is not inactivated by fluorides. In the fluorine treatment of hyperthyroids, organically bound fluorine appears to be most effective. If these substances are administered in small doses over a long period, basal metabolism is little affected; body weight, on the other hand, rises steadily. Important is the fact that, by means of fluorine therapy, the liver, also the muscles, are protected from the hyperthyroidal loss of glycogen and counteract the rapid onset of fatigue in the patient. Fluorine preparations are slower in action than thyreostatic drugs, but they can be used together. Whether the antithyroidal protective substance contains fluorine, as Kraft suggests, is at present difficult to say.

In spite of the outstanding success achieved with iodized table salt in regions of endemic goitre, there are authorities for whom the success of prophylaxis with iodine is not incontrovertible evidence for the *iodine-deficiency theory of goitre.* Eugster (1952) argues rightly against the recruiting statistics obtained in Switzerland, in particular. He is entirely correct, because in these statistics operated goitres are classified as non-goitrous, so that, according to the requirements of recruiting, the goitrous subjects may be discharged or, on another occasion, recalled to military service. Precisely these particulars of previously-removed organs are not covered by the statistics. Eugster, along with Dieterle (1952), has recorded in detail his investigations in Spain. The iodine-deficiency theory could in no ways be confirmed. Gross surgical goitres were observed near the coast, whereas there was no endemic goitre in the highlands where the iodine content of soil and drinking water was low. Eugster and Dieterle have also carried out experiments in certain parts of the canton Aargau; they come to the conclusion that goitre is to be regarded as an environmental disease. In some villages, even within the same district, are found groups free of goitre.

If we are not yet clear about the way iodine works, though it has been studied for a considerable time, the same is even more true for fluorine, which has been a matter of interest for only about the last thirty years. Some aspects are by no means clear, fluorine appearing not to work constantly and everywhere in the same manner; but one thing is certain, *between the iodine content and the fluorine content of the thyroid and the blood there is an antagonism depending on dose levels; this has to be considered.*

Our idea of the antagonism between fluoride and iodide has recently been further confirmed by Jentzer (1959). He used rabbits of equal weight from the same litter. Some of these were given 0·05 mg of fluoride per day, orally. The levels of organic iodide in the thyroid were measured by a method utilizing the Kjeldahl apparatus. Results were as follows (Table 9.11)—

TABLE 9.11

Rabbits	mg/day of *fluoride*	Duration of treatment (*weeks*)	mg of organic iodide per 100 g *of thyroid*
Control Fluoride	0·05	6	20·2 14·2
Control Fluoride	0·05	10	20·8 16·4
Control Fluoride	0·05	12	23·7 14·9
Control Fluoride	0·05	18	23·0 17·1

Thus, fluoride decreases the formation of thyroxin in the thyroid; the values found for the level of organic iodide in the thyroid are regularly less than those found in the control rabbits.

Jentzer was able to show that in the utilization of ^{131}I the localization of the radioactivity takes place in three stages:—

First stage: localization of "ionized iodide" in the thyroid gland.

Second stage: synthesis of thyroxin in the thyroid.

Third stage: entry into the blood of thyroxin, which is then partly localized in the posterior hypophysis, as hypothesized by Courrier (1949).

The rabbits treated with fluoride, as well as the controls, then received an intravenous injection of 2 mc of ^{131}I. The animals were sacrificed, and radiographs (Plate XIV (*c*) and (*d*)) were made of their hypophyses.

XII. SUMMARY

It should be emphasized that we do not in any way intend to question the anticariogenic action of fluorine. We can take it as a fact, well established according to the literature, that fluorine has a 30 to 40 per cent protective action against caries, though this action is manifest only in young people up to the age of 16 to 18 years.

The real question posed is whether there can be any justification for forcing fluorine on a whole population of which only a part can benefit from it. There can be no contracting out from fluoridized water, and it makes compulsory the intake of fluorine from all food and all drink. This could be justified only if we were concerned with an inert material, which is far from being the case. All authorities agree that fluorine is in no way an innocuous substance; moreover, since its therapeutic range is so very small, it easily lends itself to overdosage.

Fluorine is only slowly excreted and can accumulate in the body. It is laid down in bones and teeth, and there the antagonism between it and calcium must come into action. Fluorapatite is formed in the bones, but we are unable to assert that the possible amounts of fluorapatite can in fact produce denser or more resistant bone. There is a true antagonism between fluorine and the amounts of iodine taken up by the thyroid. This may result in an approximately 20 to 30 per cent reduction in function.

The thyroid gland in Switzerland cannot be fairly compared with the gland of plain dwellers. In Europe, where extremely large aggregations of population are not involved, general fluoridation of water could be carried out only in the large towns; in the country, particularly in Switzerland, any universal fluoridation of water is impossible, since every farm has its own well. This is one of the reasons why fluoridation of water has been little practised in Europe, and why the same importance has not been there attributed to fluorine as in the United States.

Because of its varied activities, there can be no question of anything but an accurately measured supplement, and in no circumstances an addition to drinking water, which makes impossible any kind of exact dosage and control.

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